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The Role of Human Papillomavirus in Cervical Disease

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**A Thesis submitted for the Degree of Doctor of Philosophy
The University of Edinburgh
May 2002**



ABSTRACT

The work presented in this thesis was undertaken to investigate the role of human papillomavirus (HPV) testing for improved detection of cervical neoplasia in the Scottish Cervical Screening Programme (CSP).

A longitudinal study of 975 archived cervical smears from women who had histologically-confirmed cervical intraepithelial neoplasia (CIN) showed HPV positivity using Hybrid Capture Assay II (HCAII) to increase with increased cytological abnormality. 18% of smears overall contained high-risk HPV (HR-HPV) DNA. Retrospective analysis of the associated cytology data showed that the positive predictive value (PPV) of cytology alone for the detection of significant cervical disease was 70.5% while that of HPV testing alone was 39%. Combined cytology and HPV testing before biopsy would have increased the PPV to 78%. However, the negative predictive value (NPV) of a negative HR-HPV result with normal cytology was 100%. These results suggested that HPV testing may have a greater role in the exclusion of severe cervical disease rather than as a predictor of its presence.

In a split-sample study, comparing conventional Pap and liquid-based cytology (LBC), monolayer preparations from LBC samples reduced the inadequacy rate from 7.3% to 2%. LBC also reduced the number of borderline smear reports from 7.9% to 4.2% and increased the number of severe cytological abnormalities detected. The clinical value of LBC was further endorsed in a direct-to-vial study, where LBC replaced conventional smears within the CSP. Monolayer preparations from over 4700 LBC samples showed a reduction in the inadequacy rate from around 10% to less than 1%. This reduced the level of anxiety in women and provided savings in repeat clinic visits for patients, repeat samples for laboratories and administrative time at both sites.

HPV testing would be a useful adjunct to cytology for improved management of women with borderline smear results. In the split-sample study, 44.7% of women with a previous borderline or low-grade cervical abnormality were HR-HPV DNA positive. If all 110 HPV negative women had been managed by routine recall then only two cases of CIN1 would have been missed, while 160 repeat visits and smears would have been saved. This clearly has benefits for both patients and clinicians at the primary care level and HPV-based triage has the potential to significantly reduce referrals to colposcopy.

A robotic DNA extraction system (using a BioRobot®9604) and a real-time polymerase chain reaction (PCR) assay using the LightCycler was developed as a suitable rapid diagnostic test for high specimen throughput in primary screening. Initial problems experienced with blockage of the silica columns during extraction were overcome by reduction of the volume of LBC sample processed for DNA extraction to 5mls. HPV DNA was detected in 15% of 1750 samples and in more than 80% of samples showing moderate or severe dyskaryosis. Detailed melting temperature (T_m) analysis of real-time PCR products suggested that HPV16 and HPV18 accounted for 44.3% and 15.5% of the HPV positive samples respectively. Further HPV genotyping using a Reverse Line Blot Assay (RLBA) under

development showed that multiple HPV infection was more common, with up to seven different HPVs being detected in a single sample. Thus, detailed Tm analysis of LightCycler PCR products is insufficiently specific for HPV genotyping in the clinical setting. The RLBA is a more appropriate technique for high throughput PCR product analysis. However, a single HPV test provides limited information, as HPV genotypes infecting the cervix may change over time. The clinical importance of persistent infection with different HPV genotypes is not yet clear.

There are currently no defined UK guidelines on cervical screening in HIV-seropositive women. Although 44.8% of the Edinburgh HIV cohort were HR-HPV positive by HCAII, this was not necessarily associated with the progression of cervical neoplasia. 70% of HIV-seropositive women who had at least three cervical specimens taken at approximately six monthly intervals harboured persistent HPV infection. 22 different HPV genotypes were identified by direct DNA sequence analysis, including some novel HPV genotypes whose oncogenicity has still to be determined. Only HPV type CP8304 was associated with cervical dysplasia. The work presented suggests that women with a CD4 count less than 200 cells/ μ l or HIV RNA viral load greater than 5000 copies/ml are at greatest risk of developing high-grade cervical disease. However, the results suggest that regular monitoring and effective treatment and follow-up of these women has prevented cervical disease progression to cancer and thus supports recommendations that HIV-infected women should be monitored more regularly than immunocompetent women.

In conclusion, the data presented here support the further analysis, on a large scale, of HPV testing in combination with cytomorphological assessment of cervical specimens, preferably of LBC type, to enhance the sensitivity, specificity and predictive values of the cervical screening programme, with particular relevance to the triage of borderline neoplasms and the monitoring of immunodeficient patients. It identifies the need to differentiate between high throughput HPV screening, using a test such as HCAII, and HPV genotyping necessary for diagnosis and effective clinical management in specific patient groups.

DECLARATION

The work presented in this thesis was undertaken by me as part of a research initiative at the Regional Clinical Virology Laboratory to investigate the role of human papillomavirus testing within the Scottish Cervical Screening Programme.

I declare that I alone have written this thesis and that, except where so stated, the work presented is my own.

A handwritten signature in black ink, appearing to read 'Amie-Louise Seagar', with a stylized flourish at the end.

Amie-Louise Seagar

ACKNOWLEDGEMENTS

Unfortunately I do not have the space to acknowledge everything that family; friends and colleagues have done for me over the duration of this work. Suffice to say I owe much to the following people and I wish to thank them for their individual contributions.

Many thanks to my supervisors Dr Heather Cubie and Professor David Harrison. Also to Dr Euphemia McGoogan for encouraging me to register as a postgraduate student. Special thanks to Dr Mark Arends for his support, encouragement and critical appraisal of the manuscript. What would I have done without e-mail?!

I am indebted to Mrs Pauline Pitt, Ms Gaye Gilkison and Miss Amanda Brass at the UEPD for everything from sending samples, retrieving results and keeping the link between both departments. A special thank you to Gaye for her time over the weekends, spent in front of APEX! At RCVL, I wish to thank Ms Joanne Whitehead, Mrs Catherine Moore and Dr Katie Cuschieri for their technical expertise with HPV testing methods. It has been a pleasure to work with you all.

I also wish to thank the staff at both RCVL and UEPD who have helped me out over the years. In particular, thanks to Mrs Eleanor Leslie and Mrs Linda Duncan at the RCVL for their cell culture expertise and, at UEPD, to Mr Bob Morris for introducing me to HPV plasmid work. Thanks also to Dr Bob Daziel for allowing use of the facilities at Summerhall for HPV cloning and to Dr David Stirling and Ms Anne White for performing the DNA sequencing work.

Thanks to Dr Susan McDonagh, Dr Sarah Howie and Dr Brian Watt for reviewing the manuscript and a massive thank you to Valerie Easton and Matt Whiley for their statistical expertise. More thanks to Susan and Heather for emotional support when it all got a bit much.....your words of encouragement were always greatly appreciated! Also, thanks to staff at the Scottish Mycobacteria Reference Laboratory for their support over the last year or so, especially Mrs Christine Doig. I'm still amazed I've got some of my marbles intact!

Nearly last, but not least, thanks to my friends- who have given me words of encouragement and put up with my lack of phone calls!- and a huge thank you to my family, who have always been there for me. Special thanks to my brother and father for their help with the diagrams included and the printing of this thesis- this is DEFINITELY the last time! Finally, thank you to Craig who has always kept smiling (even when I went a bit mad!), and whose support and understanding has been immeasurable.

ABBREVIATIONS AND SYMBOLS

| | |
|--------------|--|
| ALTS | ASCUS/LSIL Triage Study |
| ART | antiretroviral therapy |
| ASCUS..... | atypical squamous cells of undetermined significance |
| AZT | zidovudine |
| B/L | borderline |
| BLAST | basic local alignment search tool |
| β | beta |
| bp | base pairs |
| BSCC | British Society for Clinical Cytology |
| c72 | codon 72 |
| CCC | proline (Pro) |
| CD4 | T-lymphocyte helper cells |
| CDC | Centre for Disease Control |
| CGC | arginine (Arg) |
| CIN | cervical intraepithelial neoplasia |
| CL | CytoLyt |
| CSP | cervical screening programme |
| ddC | zalcitabine |
| ddI | didanosine |
| d4T | stavudine |
| DNA | deoxyribonucleic acid |
| dsDNA | double-stranded deoxyribonucleic acid |
| dNTPs | deoxynucleotide triphosphates |
| DNR | denaturation reagent |
| ELISA | enzyme linked immunosorbent assay |
| FDA | Food and Drug Administration |
| FRET | fluorescence resonance energy transfer |
| > | greater than |
| GP | general practice |
| GT:AT | guanosine + cytosine: adenosine + thymidine |
| GTC | guanidinium isothiocyanate |
| HAART | highly active retroviral therapy |
| HCA:- | hybrid capture assay |
| HCAI..... | first-generation hybrid capture assay |
| HCAII | second-generation hybrid capture assay |
| HCl | hydrochloric acid |
| HIV | human immunodeficiency virus |
| HPLC | high performance liquid chromatography |
| HPPTP | high pure PCR template preparation |
| HPV:- | human papillomavirus |
| HR-HPV | high-risk human papillomavirus |
| LR-HPV | low-risk human papillomavirus |
| HSV | herpes simplex virus |
| HTA | Health Technology Assessment |
| hTERT | human telomerase reverse transcriptase |

| | |
|-----------------------|--|
| ICTV | International Committee on the Taxonomy of Viruses |
| INAD | Inadequate |
| IPTG | isopropyl β -D-thiogalactopyranoside |
| IVDU | intravenous drug use |
| kb | kilobase pairs |
| < | less than |
| LBC | liquid-based cytology |
| LC | Lightcycler |
| LiPA | line probe assay |
| LLETZ | large loop excision of the transformation zone |
| LREC | Lothian Research Ethics Committee |
| ml..... | millilitre (10^{-3} litre) |
| mm ³ | cubic millimetre |
| MCS | multiple cloning site |
| Mod | moderate |
| NCBI | National Centre for Biotechnology Information |
| Neg | negative |
| NHS | National Health Service |
| NICE..... | National Institute for Clinical Excellence |
| NPV | negative predictive value |
| NVP | nevirapine |
| ORF | open reading frame |
| p53 | p53 tumour suppressor gene |
| Pap | Papanicolaou |
| PCR | polymerase chain reaction |
| % | percent |
| Pos | positive |
| PPV | positive predictive value |
| RCVL | Regional Clinical Virology Laboratory |
| RFLP | restriction fragment length polymorphism |
| RLBA | reverse line blot assay |
| RLU | relative light unit |
| RNA :- | ribonucleic acid |
| mRNA | messenger ribonucleic acid |
| rpm | revolutions per minute |
| RTN | ritonavir |
| SCC | squamous cell carcinoma |
| Sev | severe |
| SDS | sodium dodecyl sulphate |
| SIL :- | squamous intraepithelial lesion |
| LSIL..... | low-grade squamous intraepithelial lesion |
| HSIL | high-grade squamous intraepithelial lesion |
| SPF-PCR | short product fragment-polymerase chain reaction |
| SSCP | single strand confirmation polymorphism |
| STM | specimen transport medium |
| TAE | tris-acetate ethylenediaminetetraacetic acid |
| Tm | melting temperature |
| TP | ThinPrep® |

| | |
|-------------|--|
| 3TC | lamivudine |
| TZ | transformation zone |
| UEPD | University of Edinburgh Pathology Department |
| UK | United Kingdom |
| μl | microlitre (10 ⁻⁶ litre) |
| μM..... | micromolar (10 ⁻⁶ molar) |
| UNG | uracil-N-glycosylase |
| U/S | unsatisfactory |
| USA | United States of America |
| UV | ultraviolet |
| WNL | within normal limits |
| X-gal | 5-bromo-4-chloro-3-indolyl β-D-galactoside |

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CHAPTER 1

INTRODUCTION

1.1 Papillomaviruses

Papillomaviruses are members of the *Papillomaviridae* family (Seventh Report of the ICTV, 2000). They are widespread in nature and infect birds and mammals. Since isolation of the first papillomavirus in cottontail rabbits (Shope, 1933), the group has expanded considerably and currently consists of more than 100 established and putative human papillomavirus (HPV) genotypes, and is still escalating. HPV infections are tropic for squamous epithelial cells and cause a variety of benign human proliferations including intraepithelial neoplasias, anogenital, oro-laryngeal and oro-pharyngeal papillomas, and other types of keratoses (zur Hausen, 1996).

1.1.1 Morphology

The papillomaviruses are small, non-enveloped, icosahedral particles that are 45–55nm in diameter. The protein coat consists of 72 capsomeres arranged in a T=7 lattice. There are two capsid proteins: one major (encoded by the L1 gene) and one minor (encoded by the L2 gene). The papillomaviruses appear morphologically very similar to the polyomaviruses, but there is no nucleic acid sequence relatedness between these two groups (Pfister, 1984).

1.1.2. Genome

The papillomavirus genome consists of circular dsDNA which has a molecular weight of 5×10^6 Da and is approximately 8kb in size. The genome can be divided into early (E) and late (L) regions containing at least six early and two late open reading frames (ORFs). The function of these is outlined in Table 1.1. Messenger RNA (mRNA) is transcribed from the ORFs that are located on only one DNA strand of the HPV genome. Due to the small HPV genome size, the ORFs are distributed with considerable overlap.

| Gene | Function |
|------|---|
| E1 | Initiation of viral DNA replication. ATPase–dependent helicase activity for unwinding DNA and acts as an elongation factor for replication. |
| E2 | Encodes two separate proteins: a full–length protein product (transcriptional activator) and spliced mRNA short protein (transcriptional repressor) required for DNA replication. Also involved in maintenance of episomal state of the virus by inhibiting expression of E6 and E7 from their promoters. |
| E4 | Late protein. Interacts with the cell intermediate filament network (cytokeratins) causing them to collapse producing the cytoplasmic halo effect known as koilocytosis. |
| E5 | Membrane transforming protein. Interacts with cell membrane receptors such as EGFR to activate mitogenic signalling pathways (important in E6–associated p53 degradation). |
| E6 | Cellular transformation. Can bind to p53 tumour suppressor gene and bring about p53 degradation. |
| E7 | Cellular transformation. Forms complexes with retinoblastoma tumour suppressor gene (pRb) and Rb–related proteins p107 and p130. |
| L1 | Major capsid protein |
| L2 | Minor capsid protein |

Table 1.1 Functions assigned to the papillomavirus open reading frames (reviewed in Scheffner *et al.*, 1994 and Howley, 1996).

The other DNA strand appears to be non-coding due to the presence of widespread termination codons in all three reading frames. During replication, complex transcriptional and splicing events occur thus producing a far greater number of transcripts from the ORFs. In addition, between the early and late genes there is a non-coding region known as the long control region (LCR) or upstream regulatory region (URR). This contains promoter and enhancer elements for the regulation of transcription from the early and late genes, thus controlling the production of viral proteins, viral DNA replication and new viral particle formation (Turek, 1994).

1.1.3 HPV classification

On a genetic level, mucosal and cutaneous papillomaviruses are subdivided into genotypes and subtypes based on the extent of their DNA sequence relatedness, which can then be summarised using a phylogenetic tree (Figure 1.1). In the past, an HPV isolate was classified as a new type if it had less than 50% hybridisation in the liquid phase with any other known HPV genome (Coggin and zur Hausen, 1979). Now, however, a new genotype is defined when an isolate has less than 90% sequence homology in the E6, E7 and L1 ORFs compared to other known HPV types (de Villiers, 1994). A subtype has 90–98% homology and a variant has greater than 98% homology.

Of the 83 classified HPV genotypes (18th International Papillomavirus Conference, Barcelona, 2000), more than 40 are known to infect the genital epithelium. Such anogenital HPV genotypes are broadly divided into two groups on the basis of their oncogenic potential: low-risk or high-risk. Genital infection with 'low-risk' human papillomavirus (LR-HPV) types such as HPV 6, 11, 42, 43 and 44 commonly result in the formation of a benign outgrowth of cells known as a wart (condyloma acuminatum). These are self-limiting hyperproliferations formed by alteration in the growth pattern of the host cell when it is infected with a LR-HPV genotype. On the other hand, 'high-risk' human papillomaviruses (HR-HPVs) are strongly associated with the development and progression of preinvasive neoplastic lesions known as cervical intraepithelial neoplasia

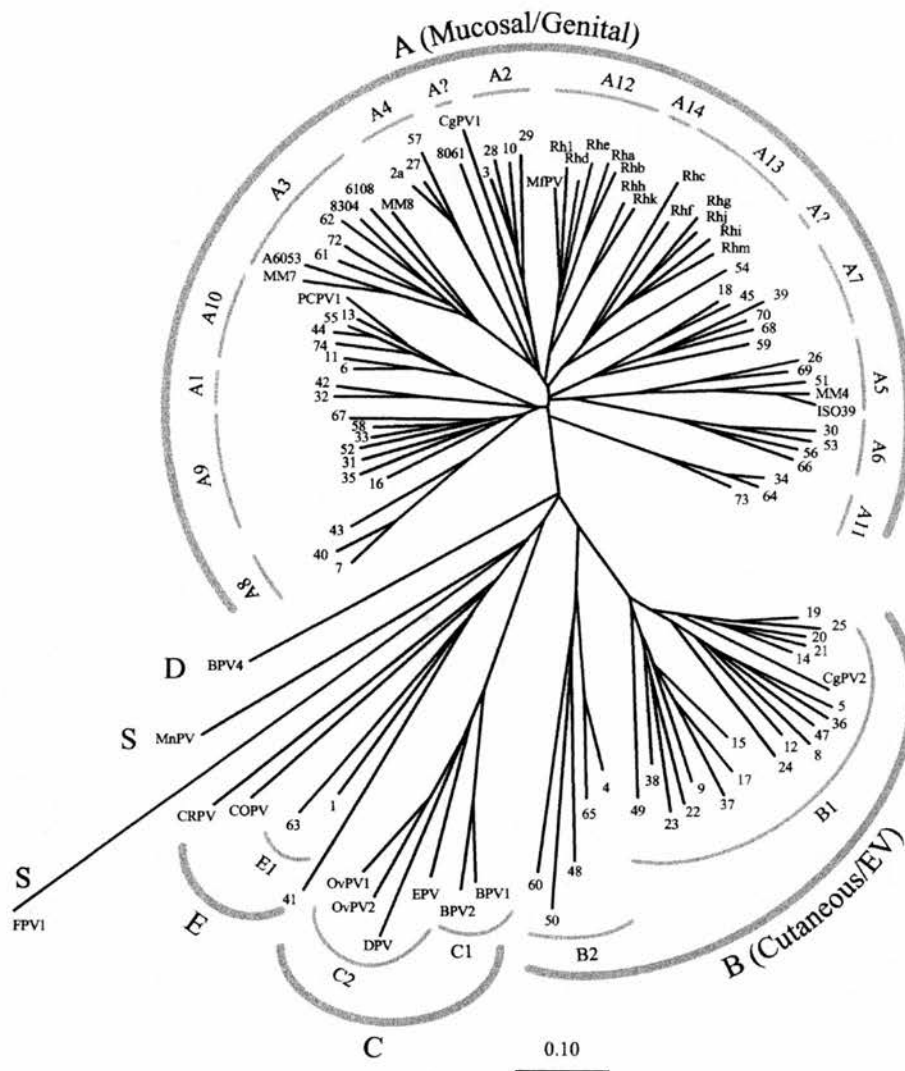


Figure 1.1 Neighbour joining phylogenetic tree of 106 papillomaviruses based on the consensus primer region of L1 (Los Alamos National Laboratory – HPV Sequence Database. Internet citation: <http://hvp-web.lanl.gov>).¹

¹ The outermost wide grey arcs show the five papillomavirus supergroups (A–E). The groups, which comprise the supergroups, are indicated by inner arcs of grey.

(CIN) and with cervical carcinoma. Such oncogenic types include HPV 16, 18, 31, 33 and 45. Indeed, women harbouring such types in the genital tract have been shown to be at greater risk of neoplastic progression from CIN to carcinoma (Schiffman *et al.*, 1993), with HR-HPV DNA detected in 97.5% of high-grade CIN and cancers (Nobbenhuis *et al.*, 1999). The extreme rarity of HPV negative cancers has reinforced the rationale for introducing HPV testing in cervical screening programmes (Walboomers *et al.*, 1997).

1.2 The Cervix

The cervix is the lower part of the uterus that projects into the vagina. It is normally covered by a non-keratinising stratified squamous epithelium, which is well adapted to withstand the normal vaginal environment. This epithelial layer is continuous with the vaginal squamous epithelium and with the simple, columnar mucus-secreting epithelium of the endocervical canal. The junction between stratified squamous and columnar epithelium normally lies at the external os.

During adolescence, pregnancy or in some oral contraceptive users, the influence of ovarian hormones causes an increase in the size and shape of the cervix so that the squamocolumnar junction is exposed to the vaginal environment, forming a 'cervical ectropion'. Thus, the endocervical columnar epithelium is exposed to the acidic vaginal environment which causes the highly specialised, fragile columnar cells to become replaced by more resistant stratified squamous epithelial cells, by a process called metaplasia. This area of metaplastic squamous epithelium is indistinguishable from the vaginal epithelium and is referred to as the transformation zone (TZ). The TZ appears to be unstable and susceptible to dysplastic changes induced by external factors. This area is the most common site for the development of cervical intraepithelial neoplasia and invasive carcinoma.

1.3 Pathogenesis of HPV Infection and the Role of HPV Proteins in the Development of Cervical Neoplasia

HPV infects the basal cells of the squamous epithelium, possibly through small abrasions in the tissue. The virus life cycle is then closely associated with keratinocyte differentiation. In premalignant cervical lesions the HPV genome exists in a free, extrachromosomal, circular form or 'episome' (Park *et al.*, 1995). Viral genome amplification and gene expression increase as the infected cell undergoes progressive differentiation until late gene expression and viral production occurs in the terminally differentiated superficial cells. This form of infection leads to benign morphological changes, such as koilocytosis, and possibly mild dyskaryosis (Southern and Herrington, 1998). The virus is shed from epidermal cells when they are sloughed off and can be transmitted by sexual or non-sexual routes.

However, in many (75–81%) cervical cancers the viral DNA integrates into the host cell genome (Park *et al.*, 1997; Pirami *et al.*, 1997) and a consistent, though sometimes low, copy number of the viral genome is maintained in transformed cells. Although no specific chromosomal binding site has so far been identified, the integration event frequently causes disruption or deletion of the E2 open reading frame (involved in transcriptional control). This leaves viral transforming oncogenes E6 and E7 directly coupled to the viral promoter and enhancer sequences of the LCR, which results in increased expression of HR-HPV E6 and E7 genes (zur Hausen, 2000). E6 encodes a protein that binds to and induces degradation of the p53 tumour suppressor gene by proteolysis (Scheffner *et al.*, 1990). The lack of p53 function within the cell increases susceptibility to mutation and chromosomal instability. E7 oncoproteins bind to the retinoblastoma tumour suppressor gene product (pRb) and other retinoblastoma-related proteins (Dyson *et al.*, 1989). Interference with the normal function of pRB influences cell cycle control (from G1 to S phase) resulting in increased transcription of genes necessary for DNA replication (Turek, 1994).

1.4 Risk Factors for Cervical Neoplasia and Cervical Cancer

1.4.1 Introduction

Cervical cancer is the second most common cancer in women worldwide and the eighth most common cancer in European women (Muir and Boyle, 1990). HPV infection is the single most important risk factor for the development and progression of cervical neoplasia. Clinical HPV infection results in lesions that can be detected by Papanicolaou (Pap) smear, colposcopy and histology. However, other forms of HPV infection are possible: subclinical infection requires identification by colposcopy (examination of the cervix using relatively low, stereoscopic magnification and bright illumination) or by cytological or histological assessment and latent HPV infection produces no clinical symptoms and evidence of infection can only be detected using nucleic acid detection methods. In the majority of women HPV infection is either latent or subclinical.

The prevalence of HPV infection has been estimated in many studies resulting in wide variation in such estimates from a few percent up to over 30%. Furthermore, cervical HPV infection is influenced by age with particularly high prevalence in women under 25 years of age. In women aged 18–30 years HPV prevalence ranges from 21% to 40% (Bauer *et al.*, 1991; Evander *et al.*, 1995; Ho *et al.*, 1998), which probably reflects increased sexual behaviour in this population. In most areas, HPV incidence then declines consistently with age gradually dropping to 2–5% in women over 35 years (Cuzick *et al.*, 1998). Interestingly, however, in a Costa Rican study a clear increase in HPV incidence rate was shown in women after the menopause (Herrero *et al.*, 2000). Although this observation warrants further study, it may indicate effects caused by changes in sexual behaviour or possible reactivation of latent HPV infection in relation to ageing of the immune system after the menopause.

To complicate matters even further, HPV infections have the potential to regress, persist or progress. Most HPV infections are self-limiting and will spontaneously regress within a few

months (median clearance time of 8 months in a study by Ho *et al.*, 1998). On the other hand, 30% of women with low-grade cervical intraepithelial neoplasia (CIN) will progress to high-grade CIN (CIN 3) and at least 12% (but probably many more) of CIN 3 cases will progress to invasive carcinoma (Arends *et al.*, 1998). So, progression along the spectrum of CIN 1–2–3 then to cervical carcinoma is not inevitable, with only a small fraction of CIN 1 cases progressing to malignancy– a process that can take many years. For example, 36% of untreated CIN 3 lesions that contain HPV DNA progress to invasive cancer over 20 years (Farthing *et al.*, 1994). Thus, the development of cervical carcinoma is not a single-step process that can be solely attributed to the presence of HPV and various other risk factors have been implicated. These include sexual behaviour indicators (including the number of sex partners and age at first intercourse), infection with other sexually transmitted agents, number of pregnancies, oral contraceptive use, cigarette smoking and immune status (Kjaer *et al.*, 1996; Temmerman *et al.*, 1999; Hildesheim *et al.*, 2001). As such, the exact mechanisms of HPV-associated cervical carcinogenesis are still only partially understood.

1.4.2 HPV genotype

There appears to be geographical variation in the distribution of HPV types found worldwide. In Europe, HPV 16 is the most prevalent high-risk genotype (Bosch *et al.*, 1995). Follow-up studies have shown it is also the single most important HPV type for predicting cervical disease progression, since HPV 16 infections start to progress earlier than other HPV types, with an estimated 80% of persistent HPV 16-positive lesions eventually progressing from a lower to a higher grade of CIN (Kataja *et al.*, 1990). Since HPV 16 appears to be a better prognostic marker of progression to high-grade cervical neoplastic lesions than other HPV genotypes (Cuzick *et al.*, 1994), research to identify women at highest risk of the development of cervical cancer has so far focused on detection of this genotype. Specifically, HPV 16 has been detected in 30–77% of women with CIN 2–3 in various studies (Schiffman *et al.*, 1993; Gaarenstroom *et al.*, 1994; Munoz and Bosch, 1996) but is found less frequently in women with CIN 1 (Cuzick *et al.*, 1994). In addition, women harbouring HPV 16 in association with normal or mildly abnormal cytology are also more likely to have a more severe underlying histological lesion (Cuzick *et al.*, 1994).

The second most common high-risk genotype, HPV 18, is associated with the development of a more clinically aggressive or more advanced disease (Kurman *et al.*, 1988; Burger *et al.*, 1996; Arends *et al.*, 1993). The other prevalent HR-HPVs seen at an appreciable frequency in invasive cervical cancer are HPV 45 and HPV 31 which were found in 8% and 5% of tumour specimens tested in a world-wide study by Bosch and colleagues in 1995.

1.4.3 HPV 16 variants

HPV 16 can be classified into more than 40 variants that differ by 2% homology or less in a specified region of the genome. HPV 16 E6 has frequent sequence variations and it is suggested that some variants are associated with a higher risk of developing CIN 2–3 and invasive cancer than others due to their differential ability to induce p53 degradation (Xi *et al.*, 1997; Yamada *et al.*, 1997). It has been estimated that women harbouring HPV 16 variants are at least 6.5 times more likely to develop CIN 2–3 than those with prototypic strains (Xi *et al.*, 1997). Indeed, HPV 16 E6 variants containing a substitution at residue 83 have been detected in 88% of invasive cervical carcinomas (Zehbe *et al.*, 1998) suggesting increased oncogenicity is associated with this polymorphism. In contrast, other studies have shown no association of any HPV 16 E6 or E7 sequence variation with severity of cervical neoplasia or HPV persistence (Londesborough *et al.*, 1996; Bontkes *et al.*, 1998).

1.4.4 HPV persistence

Persistent HPV infection, often defined as the detection of the same HPV type in consecutive samples obtained at 3–6 monthly intervals, is a risk factor for the progression of a preinvasive lesion to invasive cancer. In follow-up studies of women with cytologically normal (Rozendaal *et al.*, 1996; Liaw *et al.*, 1999) and abnormal smears it has been shown that persistent HR-HPV infection of cervical cells is necessary for the development, maintenance and progression of CIN lesions (Wallin *et al.*, 1999; Remmink *et al.*, 1995; Ho *et al.*, 1995). In a study by ter Harmsel *et al.*, (1999) more women with persistent HPV 16 infection (shown by at least three consecutive PCR positive tests at six monthly intervals) were shown to develop CIN compared to women who were only transiently HPV 16-infected (shown by one positive test followed by two negative PCR results). Thus, it was

concluded that persistent HPV 16 infection is associated with a higher risk of developing cervical neoplasia, which is often high-grade. However, Mayrand *et al.*, (2000) demonstrated that HPV 16 DNA detected in consecutive samples may not indicate persistent infection at all, but re-infection with a different HPV 16 variant.

1.4.5 HPV viral load

It has also been suggested that high HPV viral load, resulting from productive viral replication, is a marker for persistent infection and thus an increased risk for the development of cervical dysplasia. Hence, estimates of viral load may be useful in selecting for treatment only women with high levels of viral DNA that are likely to persist or confer an increased risk of cervical disease progression.

Recent studies have shown that HPV DNA quantitation can predict the risk of developing cervical cancer much earlier than other screening methods. In particular, high HPV 16 viral load has been shown to increase the relative risk of carcinoma *in situ* by at least 30 times (Ylitalo *et al.*, 2000). Women with persistently high HPV DNA load are most at risk of cervical disease progression. Women under 25 years of age are particularly at risk since 25% with persistently high HPV DNA load will develop cervical malignancy within 15 years (Ylitalo *et al.*, 2000). In a study of archival smear material, Josefsson and co-workers (2000) produced similar findings and concluded that women with the 20% highest amount of HPV 16 DNA were 60-fold more at risk of developing cervical carcinoma *in situ* than HPV negative women.

Many studies have attempted to estimate HPV DNA load from the signal intensity produced by commercially available hybridisation tests. However such assays were designed only to show simply the presence or absence of HPV infection, thus any viral load estimation made in this way is not reliable. Indeed, the grade of disease, the number of infected epithelial cells present in the clinical specimen and the distribution of viral copies will all influence the HPV viral load detected, which may even be different for individual HPV types.

Additionally, the detection of just a few copies of HPV DNA may not be relevant for subsequent cervical disease. With the advent of real-time PCR, quantitative assays have been developed for accurate determination of HPV viral load in cervical specimens (Johnston, 2000), but a specific viral load cut-off that defines women at high-risk of disease progression has not yet been determined.

1.4.6 Immune status

1.4.6.1 General immunosuppression

Cellular immunodeficiency is considered to be an important risk factor for HPV infection and persistence and for the progression of CIN (Petry *et al.*, 1994). Skin carcinomas, which are often preceded by viral warts and premalignant keratoses, are particularly well-recognised in patients following renal transplantation (Berkhout *et al.*, 2000). Female renal transplant recipients are also at risk of genital HPV infection and subclinical cervical HPV infection and cervical dysplasia, in particular with HPV types 16 or 18 (Brown *et al.*, 2000). Approximately 15 years or more after receiving a renal allograft nearly all recipients will have evidence of HPV infection and it is notable that cervical or ano-perineal cancers represent some of the potentially fatal complications of both immunosuppression following organ transplantation and HIV infection (Benton and Arends 1996; Arends *et al.*, 1997).

1.4.6.2 HIV-associated immunosuppression

Studies have consistently shown that HIV-infected women have an increased prevalence of anogenital HPV infection with increased development of CIN and cervical cancer compared to HIV-seronegative women (Schrager *et al.*, 1989; Feingold *et al.*, 1990; Vermund *et al.*, 1991; Marte *et al.*, 1992; Branca *et al.*, 1995; Sun *et al.*, 1997; Williams *et al.*, 1994; Wright *et al.*, 1994a). In addition, this immunocompromised population is subject to a higher rate of CIN recurrence and treatment failure (Maiman *et al.*, 1993). The risk of HPV infection and the severity of cervical neoplasia has been linked to

impaired cell mediated immunity, as measured by CD4 lymphocyte counts (Maiman *et al* 1993; Johnstone *et al.*, 1994; Vernon *et al.*, 1994).

HIV-seropositive women have an eight-fold higher risk of HPV-related cervical disease than HIV non-infected women (Capiello *et al.*, 1997). Depending on the level of immunosuppression, such HPV infections are approximately seven times more likely to be persistent infections (defined as the detection of the same HPV at two or more examinations during a period of 3–12 months) (Sun *et al.*, 1997). In addition, HIV-infected women have a higher percentage of infections with multiple HPV types compared to HIV non-infected women with two or more high-risk HPV types being commonly found (Shah *et al.*, 1997).

Invasive cervical cancer was added to the list of CDC AIDS-defining illnesses in 1993 (Centres for Disease Control, 1992; Klevens *et al.*, 1996), but preservation of immune function by the introduction of highly active antiretroviral therapy (HAART) may reduce the risk of cervical disease in HIV-seropositive women. Alternatively, if HAART has little or no impact on the regression of cervical disease then cervical neoplasia may become increasingly common as patient survival is prolonged. However, it is too early to assess fully the impact of HAART on the natural history of CIN in HIV-infected women. Early data suggests that successful suppression of plasma HIV viral load using antiretroviral drugs may reverse immunosuppression resulting in regression of CIN lesions despite the presence of persistent HPV infection (Heard *et al.*, 1998). Further studies are required to confirm these findings.

1.5 Host Cell Genetic Alterations in HPV Infection and Cervical Neoplasia

1.5.1 p53 tumour suppressor gene

p53 function plays a key role in DNA repair. In the normal situation, it is present in a very low concentration in cells but when DNA is damaged, its concentration increases. It then

acts as a transcription factor inducing the expression of an inhibitor that leads to cell cycle arrest in both G1 and G2. This allows DNA repair if possible or apoptosis and cell death if the damage is severe. Degradation of p53 by HR-HPV E6 binding is of central importance in tumourigenesis as loss of p53 function increases susceptibility to mutations in the host cell DNA. Storey *et al.*, (1998) showed that host p53 protein was polymorphic at codon 72 (c72) in that the p53 protein contained either an arginine (CGC) or a proline (CCC) residue at this position (within exon 4). This was the first evidence that there was a possible genetic susceptibility to the development of squamous carcinoma of the cervix among white women since polymorphic variants differed in their susceptibility to degradation mediated by the HPV E6 oncoprotein. The E6 proteins from HR-HPV types were more effective at degrading the arginine form (p53Arg) than the proline form (p53Pro). Storey and colleagues found that patients with HPV-associated cervical cancer were much more likely to have the p53Arg form and estimated that patients with two copies of the arginine form had a 7-fold increased risk of developing cervical cancer than individuals with two copies of the proline form.

Although several groups worked speedily to support these findings using larger case-control studies, they were unable to confirm the association between the p53 Arg polymorphism and cervical neoplasia (Hildesheim *et al.*, 1998; Helland *et al.*, 1998; Josefsson *et al.*, 1998). Rosenthal and colleagues (1998) examined the distribution of the p53 polymorphism in a similar group of white women (50 cases and 246 controls) but did not find a significant excess of c72 arginine alleles. In another UK-based study (Giannoudis *et al.*, 1999), no significant differences were identified in the frequencies of p53Arg genotype in women with varying degrees of dysplasia (equivalent to 118 LSIL, 118 HSIL and 43 invasive cancers) and without cervical disease (n=30). Additionally, there was no significant association between p53 genotype and infection with HR- or LR-HPV types. In a larger case-control study of 205 women (Sonada *et al.*, 1999), the frequency of individuals homozygous for arginine at c72 was lower in the squamous cell carcinoma cases but higher in the control group than the findings by Storey and colleagues. The role of p53 c72 polymorphism was

also examined in women with premalignant disease (353 cases and 496 controls) by Lanham *et al.*, (1998) but no evidence was found to show that the arginine genotype at c72 of p53 was involved in the progression of HPV-associated cervical malignancy. Szarka *et al.*, (1999) performed a study in Hungarian women that showed that the p53Arg homozygous genotype does not increase the risk of cervical carcinoma specifically in HPV16-infected women.

Possible reasons have been advanced to explain why the results of Storey *et al.*, (1998) cannot be reproduced in other investigations. The aforementioned study involved only a small number of women with cervical cancer (n=30) and slightly more controls (n=41). The results generated therefore may have been influenced by the small sample size. In addition, it has been reported that the frequency of the p53 Arg/Pro polymorphism varies with ethnic group (Beckman *et al.*, 1994). Elderly oriental women (60–96 years) with cervical carcinoma showed a higher proportion of p53 point mutations (11%) caused by endogenous mutagenesis (Nakagawa *et al.*, 1999), although this result fell short of statistical significance. In contrast however Yamashita *et al.*, (1999) reported no relationship between p53Arg and predisposition to HPV-associated cervical neoplasia in their study of HPV-infected SIL and invasive cancer cases from Japanese women compared to a control group.

1.5.2 Telomerase

The analysis of telomerase expression may provide another valuable tool for the detection of neoplastic cells. Telomerase is an enzyme that allows the maintenance of telomere sequences following DNA replication. Telomeres act as protective caps at the ends of chromosomes. Every time a cell divides it loses 50–100bp at the ends of its telomeres. When a critical number of base pairs are lost, it triggers a signal for the cell to stop dividing and senesce. Activation of a specialised reverse transcriptase (telomerase) allows cells to avoid this progressive shortening of telomeres by adding back the telomere sequences that are lost, therefore allowing cells to multiply indefinitely. HR-HPV E6 induces telomerase

activity by increasing the expression of the telomerase catalytic subunit, human telomerase reverse transcriptase (hTERT) (Weinberg, 1998). This action is independent of E6–p53 degradation (Klingelhutz *et al.*, 1996). Degradation of p53 by E6 may however be important at a later stage in neoplastic progression as loss of p53 function increases susceptibility to mutations in the host cell DNA. Nevertheless, telomerase activity alone is not enough to immortalise keratinocytes and inactivation of the retinoblastoma tumour suppressor pathway (that regulates transition from G1 to S phase of the cell cycle) by E7 is also required (Kiyono *et al.*, 1998).

Snijders *et al.*, (1998) investigated telomerase activity and hTERT mRNA expression in relation to HR–HPV DNA presence in women with precancerous cervical lesions. hTERT mRNA levels were strongly associated with detectable telomerase activity and these were only detected in cases that harboured high–risk HPV DNA. It was concluded that telomerase activity with an accompanying increase in the level of hTERT mRNA, reflected a rather late stage in the progression of CIN to carcinoma which follows HR–HPV infection.

1.6 The Cervical Screening Programme (CSP)

Cervical screening was first introduced in Britain in 1967, although it was not initially introduced as a population–based programme. Now cervical screening is well established throughout the National Health Service (NHS) and aims to reduce the mortality rates associated with cervical cancer by early detection and treatment of pre–malignant disease. Every year 360,000 women between 20–64 years are invited, free of charge, for cervical screening. Computerised call and recall was introduced in 1988 and women receive an invitation every 3–5 years (Patnick, 2000). The current cervical screening procedure involves standard cytological assessment of epithelial cells from the cervical transformation zone by a conventional Pap smear and if appropriate colposcopic assessment with cervical biopsy or large loop excision of the transformation zone (LLETZ).

Since the introduction of the Pap smear, widespread cervical screening in industrialised countries has reduced cervical cancer incidence rates by 70% or more to approximately 10 per 100,000 per year. In developing countries however, possibly due to the lack of successful Pap smear screening programmes, the cervical cancer incidence rate is more than 40 per 100,000 per year and mortality is high (Lörincz, 1996). Despite an 87% screening coverage in Scotland, cervical cancer still remains the 10th most frequently diagnosed cancer with 122 deaths reported in 1999 (internet citation: <http://www.nhsis.co.uk>).

1.7. Cytological Assessment of Cervical Neoplasia

Specimens for conventional Pap smear preparation are collected from the cervix using either an Ayre or Aylesbury® spatula (CellPath, Powys, Wales) and as much of the cellular material as possible is transferred onto the surface of a glass slide. The cells are fixed and the slide is transported to the laboratory where it is stained with Papanicolaou stain. Microscopic assessment of the epithelial cells can then be performed. Cytological techniques can provide suggestive evidence of HPV infection by identification of koilocytes; non-neoplastic epithelial changes caused by the viral cytopathic effect. Koilocytes are abnormal squamous cells with characteristic perinuclear vacuolation, which show irregular but clear edges bounded by dense cytoplasm (Figure 1.2). They are the hallmark of productive HPV infection. However, such morphological changes are not specific or sensitive for oncogenic HPVs and are more likely to detect LR-HPV types that produce benign lesions.

1.7.1 Cytology classification schemes

The diagnostic terminology applied to cervical cytological abnormalities has undergone several changes over time, which can sometimes lead to confusion in the literature.

In the UK, neoplastic changes in the squamous epithelium detected by cytological assessment of Pap smears are described using terminology introduced by the British Society for Clinical Cytology (BSCC) in the 1980s. This system describes preinvasive neoplastic

lesions of the cervix that can advance through various stages of dysplasia, followed by carcinoma in situ and then invasive carcinoma and attempts to correlate the histological findings with appropriate cytological abnormalities. The classification scheme uses the term 'dyskaryosis', which can be divided into mild, moderate or severe categories according to the degree of severity of nuclear atypia and other changes. These correlate with the histological grades of CIN 1, 2 and 3 respectively. The main observation of dyskaryosis is the enlarged nuclear: cytoplasmic ratio that increases as the lesion progresses. The BSCC terminology uses five grades in total: 1. Unsatisfactory for assessment (with a reason stated), 2. Negative or within normal limits (WNL), 3. Nuclear changes bordering on mild dyskaryosis, also called 'borderline' (B/L), 4. Dyskaryotic cells: mild, moderate or severe, 5. Malignant cells suggestive of invasive cancer (squamous or adenocarcinoma).

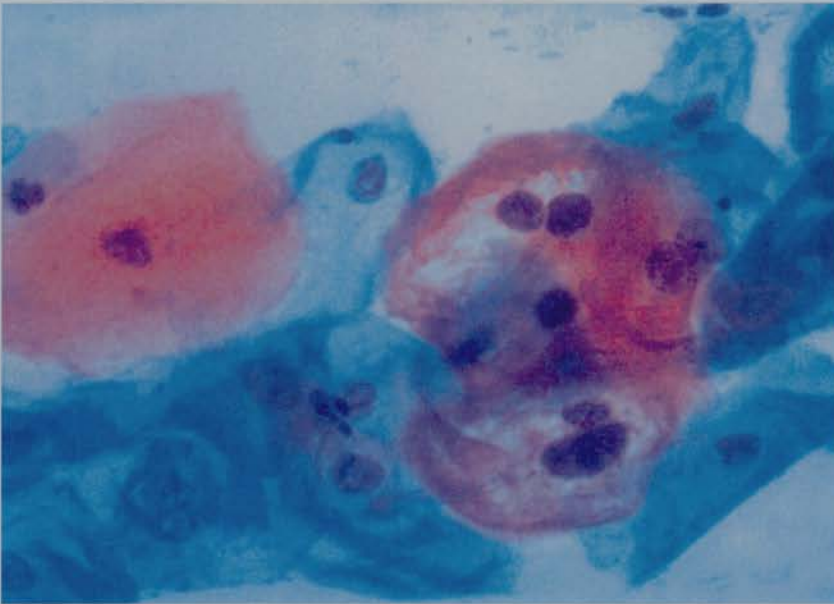


Figure 1.2 Conventional Pap-stained cervical smear showing koilocyte development (provided courtesy of Dr E. McGoogan).

In the USA, the Bethesda System for Reporting Cervical/Vaginal Cytologic Diagnoses (National Cancer Institute Workshop, 1989) is widely adopted to describe cytological abnormalities. This system classifies precancerous lesions under the term squamous intraepithelial lesion (SIL), which is subdivided into two categories: low-grade squamous intraepithelial lesions (LSIL) and high-grade squamous intraepithelial lesions (HSIL). There is also a further category of atypical squamous cells of undetermined significance (ASCUS).

1.7.2. New technologies for cytological assessment

An alternative approach for improved cytomorphological detection of cervical neoplasia is liquid-based cytology (LBC). This requires the sample that is removed from the cervix being rinsed into a vial of preservative fluid, which is then used to prepare a thin layer smear preparation using automated technology operated by skilled laboratory staff. These slide preparations show optimal cell preservation, reduced debris from mucous, inflammatory cells or blood and minimal cell overlap. Two such devices are AutoCyte PREP (AutoCyte, North Carolina, USA) and ThinPrep® 2000 (Cytoc Corporation, Maryland, USA). The added advantage of LBC is that only a proportion of the cells in the cervical scrape sample are used to produce the thin layer preparation and so the residual cells can be used for HPV testing without requesting another patient visit.

Several studies have positively reported on the utility of LBC for improved cervical screening (Dupree *et al.*, 1998; Austin, 1998; and others discussed in Section 6.4.1) and the feasibility of ThinPrep®-HPV combination testing as a replacement or adjunct to the conventional Pap smear has been investigated (Sherman *et al.*, 1997). Improved LBC preparations result in reduced smear screening and interpretation time while showing comparable or increased detection of cervical abnormalities in comparison with conventional Pap smears (Aponte-Cipriani *et al.*, 1995; Bolick and Hellman, 1998; Lee *et al.*, 1997). While the cost of reagents and hardware is more expensive for liquid-based cytology it is argued that long term cost savings are likely due to the reduction in both the

number of inadequate and equivocal smear reports. In addition, LBC has been shown to be useful for identifying women with B/L smears who have an underlying CIN lesion (Ferris *et al.*, 1998; Crum *et al.*, 1999; Manos *et al.*, 1999). By reducing the frequency of repeat Pap smear testing and colposcopy referrals, costs will be reduced even further and, in turn, the prospect of increasing the screening interval from every three years for some women will bring additional cost savings.

Computerised automated cytology screening devices that use advanced neural network technology to assess the degree of cellular abnormality present in a cervical smear preparation are now available. One such system, called PapNet (Netherlands B.V., Zekeringstraat, Amsterdam) has two components: a scanning machine and a review platform. Initially, Pap-stained smears are loaded onto a motorised microscopic stage where an electronic camera digitises 128 images from the most abnormal cells in each smear, which can be stored on disk. The cytoscreener can then focus on these areas on a personal computer to obtain a rapid and reliable impression. Slides requiring further microscopic review can then be fully microscopically assessed by a cytotechnologist. Initial evaluations of the system compared to conventional methods of screening have been positive (Bosanquet *et al.*, 1999).

1.8. Histological Assessment of Cervical Neoplasia

When significant dyskaryotic changes have been identified cytologically, patients are referred for colposcopic assessment and histological examination of biopsy specimens taken at colposcopy is used to define accurately the degree of dysplasia and to plan appropriate treatment. Colposcopy involves examination of the cervix using relatively low power, stereoscopic magnification and bright illumination. This method has a high sensitivity (>90%) for the detection of high-grade disease but also has low specificity (<50%) that results in a high rate of over treatment. Cervical neoplasia assessed histopathologically is described as CIN which, in Europe, is classified into three grades

according to the degree of severity. CIN 1 and 2 correspond to mild and moderate dysplasia respectively and CIN 3 includes both severe dysplasia and carcinoma in situ. Preinvasive lesions are confined to the surface cervical epithelium and underlying crypts but do not invade through the basement membrane into the underlying connective tissues. With any evidence of invasion through the basement membrane into the underlying tissues, the lesion is defined as invasive or microinvasive squamous carcinoma of the cervix.

1.9. Adenocarcinoma

Squamous carcinomas account for a high proportion of cervical malignancies with the remainder being made up mostly of adenocarcinomas arising in the endocervix. Adenocarcinoma is preceded by dysplastic changes in the endocervical glandular epithelium termed cervical glandular intraepithelial neoplasia (CGIN) which often co-exists with squamous CIN. Either or both the surface epithelium and the underlying crypts may be affected. The current grading system for CGIN divides lesions into high grade (including adenocarcinoma in situ and severe glandular dysplasia) or low grade (moderate and mild glandular dysplasia). The association between CGIN and HPV is not as strong as for squamous CIN, but frequently adenocarcinomas are associated with HPV DNA, particularly HPV 18 (Arends *et al.*, 1998). It has been suggested that glandular tumours of the cervix are more aggressive than squamous cervical tumours (Hildesheim *et al.*, 1999).

1.10 Limitations of the NHSCSP

Despite great efforts to improve the coverage of the NHSCSP, still only 85% of eligible women attend their screening appointments. In some countries, recent studies have focused on methods of self-sampling as a way of improving the population coverage and also as a cost-effective approach for screening in under-developed countries which lack organised screening programmes. So far, a good correlation (85–93%) has been

reported between HPV results from self-obtained vaginal samples and cervical smears taken by trained staff (Hillemans *et al.*, 1999).

More importantly, the Pap smear method itself has several limitations resulting in a high false negative rate of approximately 20% (Gay *et al.*, 1985; van der Graaf and Vooijs, 1987). A significant problem with the Pap smear method is the large number of sub-optimal and B/L smear results that are reported. Poor cell transfer or fixation of the Pap smear often occurs at the time of making the slide. Up to 90% of the cells harvested from the cervix are discarded with the sampling device and this contributes to the proportion of smears designated 'unsatisfactory' due to the lack of cells derived from the transformation zone. Such limitations were highlighted in a recent audit of the UK screening programme that found that 47% of women under 70 years of age with invasive cervical cancer had an apparently adequate screening history (Sasieni *et al.*, 1996). Similarly, invasive cervical cancer has been identified in women who had a minor cytological abnormality many years previously followed by a number of (presumably false) negative smears (Morell *et al.*, 1982; Stanbridge *et al.*, 1992). Media coverage of such unfortunate cases has resulted in some loss of public confidence in the present cervical screening process based solely on Pap smears.

It is therefore widely accepted that a screening programme based on conventional cytology alone has important limitations and new methods are required to improve both sensitivity and specificity. The main issues for consideration are the age to start and stop screening; the appropriate screening interval; the role of self-sampling and choice of primary test. Since 1993, when a survey of experts concluded that HPV DNA testing had a limited role in clinical practice (Cole, 1993), the reproducibility and sensitivity of HPV DNA tests has improved greatly. There is now substantial evidence to suggest that HPV testing would be useful in cervical screening programmes of the future although some controversy surrounds how this should be targeted for greatest effect.

1.11 Role of HPV Testing in the NHSCSP

1.11.1 Primary screening

HPV testing may be a useful adjunct to cytological assessment that could result in the earlier detection of women at risk of developing high-grade CIN or cervical carcinoma (Cuzick *et al.*, 1995; Cuzick *et al.*, 1999b). It is predicted that adding HPV testing to primary screening could increase the yield of high grade CIN by 50–100% with a positive predictive value similar to that for moderate dyskaryosis (Cuzick, 1998).

There is no doubt that universal HPV testing in all women attending cervical screening clinics would identify underlying cervical disease that was not detected by cytology. 5–10% of women with normal cytology (possibly more according to Liaw *et al.*, 1999) will harbour HPV in their cervixes which may indicate the presence of high-grade CIN (Melkert *et al.*, 1993; Kjaer *et al.*, 1996). Such women with a HR-HPV positive test at base line, are 116 times more likely to develop CIN 3 after a follow-up time of 4–6 years, than women with normal cytology and an HR-HPV negative result (Rozendaal *et al.*, 1996). However, since the population prevalence of HPV infection is high, HPV testing in primary screening would also identify women with HR-HPV infection who had no cervical disease. In particular, HPV infection in younger women, aged 18–25 years, tends to be at a high viral load but the viral infection often spontaneously regresses in this age group thus making the predictive value of a positive HPV test very low (Ho *et al.*, 1995).

In older women however, the viral load is often lower but the virus is more likely to persist (Hildesheim *et al.*, 1994). Although it is estimated that low-grade disease will regress in over 50% of cases in 3 to 4 years, persistence of low-grade cervical neoplasia (CIN 1) has also been shown to increase with age (McCance, 1998). Thus restricting HPV testing to women over 30 years of age, who have a more stable pattern of HPV infection, would substantially improve the specificity in primary screening by significantly increasing the

predictive value of a positive HPV test. Additionally, HPV testing could rationalise the current screening guidelines by extending the screening interval for women considered to be at low risk (both cytologically and HPV negative) (Rozendaal *et al.*, 2000), particularly in women over 50 years of age who have never had an abnormal smear. This would reduce the need for prolonged surveillance of women, which also has important cost implications for health care services. In the UK, the Health Technology Assessment (HTA) Committee of the Department of Health has performed a systematic review of the available evidence on the role of HPV testing in cervical screening (Cuzick *et al.*, 1999a). The findings are discussed further in Chapter 6.

1.11.2 Triage of women with borderline smears

5–10% of all smears are designated B/L as there is some doubt that they are clearly normal. The vast majority of these smear results will have no clinical relevance since the positive predictive value of a B/L result has been shown to be only 3% (Cuzick *et al.*, 1999b). Ten percent of this group however will have an underlying high-grade lesion. In women with mild dyskaryosis the underlying prevalence of high-grade CIN is much higher at up to 40% (Flannelly *et al.*, 1994). Yet a randomised study by Cruickshank *et al.*, (1999) found HPV testing using PCR to be of no significant benefit over immediate referral of women with mild dyskaryosis to colposcopy, concluding it was unlikely to be of benefit in managing women with mild dyskaryosis.

The current guidelines for women with low-grade abnormalities are unclear and B/L cytology results present a management problem since all women with this diagnosis will require follow-up to avoid missing the small percentage of clinically significant disease. Follow-up usually involves repeat cytology and/or referral for colposcopy. Present guidelines recommend referral for colposcopy after three B/L smear results or two mild abnormalities at six monthly intervals. In the Edinburgh area, approximately 60% of such women attending colposcopy are found to be within normal limits (WNL). Extrapolated to the whole of Scotland, this means that approximately 1000 women with no significant

cervical neoplasia undergo unnecessary colposcopy each year (Dr. McGoogan, personal communication). In a study of 566 women with low-grade atypia who were not treated at their first colposcopy visit, 54.1% of abnormalities resolved; 24.4% developed persistent disease and 21.5% were subsequently treated (Teale *et al.*, 2000). With an increase in the number of annual referrals for colposcopy of women with persistent borderline changes, unnecessary pressures are being imposed on colposcopy clinics and many women are being subjected to an unnecessary, psychologically stressful procedure. Their return to routine screening is also delayed.

This current procedure could be improved if an additional test could increase the detection of high-grade CIN without reducing the specificity of cytology alone. Thus, only those women with cervical disease would require referral to colposcopy and those without neoplastic changes could be followed using standard cytological surveillance. HR-HPV DNA positivity, compared to cytology alone, has been shown to improve the detection rate of high-grade cervical neoplasia in women with B/L smears (Hatch *et al.*, 1995, Cox *et al.*, 1995; Herrington *et al.*, 1995). However, this was not been confirmed in other studies where HPV testing was shown to have a low sensitivity (Kauffman *et al.*, 1997). Triage of women with low-grade cytological abnormalities using a repeat Pap smear and an HPV test was shown to have low specificity in a study by Wright *et al.*, (1995). Although the approach correctly identified 92% of women with biopsy-proven CIN, 71% of the women would have been referred for colposcopy. A large randomised multicentre clinical trial (ASCUS/LSIL Triage Study (ALTS)) has been performed in the USA to investigate the sensitivity and specificity of immediate colposcopy, repeat cytology every three months and HPV testing for the detection of CIN 3. Enrolment into the ALTS study finished in December 1998 and analysis of early data has been published (The ALTS Group, 2000). If continued studies in large populations confirm that HR-HPV detection can predict the women least likely to have high grade CIN and therefore not requiring aggressive follow up, this could alter current practice.

1.11.3 Follow-up of women following treatment for CIN

HPV testing may improve the accuracy of follow up of women who have been treated for precancerous or early invasive lesions. Currently, these women have annual smears for five years and sometimes for the rest of their lives. HPV positivity after treatment could help select those women who had failed to respond to the treatment regime or had persistent HR-HPV infection. Alternatively, the lack of HPV would identify those women whose lesions had been successfully treated and they could be returned to routine recall.

1.11.4 Management of HIV-seropositive women

As previously mentioned, women with HIV infection have an increased frequency of anogenital HPV infection with increased development of both CIN and cervical cancer. Furthermore, cervical disease recurs with greater frequency and progresses more rapidly in severely immunocompromised women harbouring HR-HPV types. On this basis, there is a definite need for adequate cervical surveillance of HIV-seropositive women (Vonau and Boag, 2000). This was highlighted in a recent study showing that HIV-infected women with no evidence of cervical disease developed histology-confirmed neoplasia within three years (Ellerbrock *et al.*, 2000). Although annual cytological screening is performed in many centres there are, at present, no defined UK guidelines on cervical screening in HIV infected women (Jungmann *et al.*, 1998). There is, however, a general consensus that HIV-infected women with relatively intact immunity (CD4 counts >500 cells/ μ l) should be treated in the same way as HIV non-infected women (Six *et al.*, 1998).

The sensitivity of the conventional Pap smear in identifying cervical abnormalities in HIV-seropositive women has been questioned. In some studies, cytology has been shown to predict accurately the presence of colposcopic or histologically confirmed disease (Wright *et al.*, 1994a; Adachi *et al.*, 1993). In other studies, the false negative rate for cytology has been shown to be as high as 23% (Tweddel *et al.*, 1994) with cytological evaluation alone failing to identify severe histological abnormalities in 42.6% of HIV-infected women (Uberti-Foppa *et al.*, 1998). HPV testing may identify

more accurately those HIV–infected women at greatest risk of neoplastic progression. Indeed, combined HPV and Pap smear testing has been shown to improve detection of histologically confirmed cervical disease (Uberti–Foppa *et al.*, 1998). HPV DNA detection has been shown to be beneficial for primary cervical cancer screening in HIV seropositive women by more accurate identification of high–grade cervical disease than the Pap smear (Petry *et al.*, 1999).

As patient survival times are increased due to the use of antiretroviral drugs, specific guidelines are needed to develop an effective programme for the early identification and management of precancerous lesions in HIV–seropositive women, particularly those who may be severely immunocompromised.

The potential applications of HPV testing within the NHS CSP are summarised in Figure 1.3.

Figure 1.3

**POTENTIAL APPLICATIONS OF HPV DNA TESTING WITHIN THE
CERVICAL SCREENING PROGRAMME**

1. ROUTINE SCREENING FOR CERVICAL NEOPLASIA

Advantages: Good sensitivity; may allow screening interval to be increased for women at low risk of cervical disease progression.

Disadvantages: Poor specificity since predictive value of an HPV positive test is very low in younger women. May only be cost effective if HPV testing was restricted to women over 30 years old.

**2. TRIAGE OF WOMEN WITH BORDERLINE CYTOLOGICAL
ABNORMALITIES**

Advantages: May identify women at risk of neoplastic progression. Potential to reduce unnecessary repeat smears and visits.

Disadvantages: Cost effectiveness unknown.

3. POST-TREATMENT SURVEILLANCE

Advantages: Could more rapidly detect lesions that have been incompletely excised. Could reduce the length of surveillance for HPV negative women.

Disadvantages: Few studies in this area– more research needed.

4. MANAGEMENT OF WOMEN WITH CHRONIC IMMUNOSUPPRESSION

Advantages: May reduce false negative cytology rates and identify more accurately women at risk of neoplastic progression.

Disadvantages: Effect of HAART on natural history of HPV and CIN is unknown.

1.12 Laboratory Identification of HPV Infection

Since HPVs cannot be readily propagated in cell cultures (due to the inability of keratinocytes to terminally differentiate *in vitro*) and only small amounts of viral antigens are present in infected exfoliated cells, the laboratory diagnosis of HPV infection requires molecular techniques such as DNA hybridisation or nucleic acid amplification. Several approaches have been used for HPV detection in cervical specimens with widely differing results. Older methods such as Southern blots, dot blots and in-situ hybridisation analyses are not as sensitive as current nucleic acid hybridisation or amplification methods or are too time consuming and labour intensive for widespread application.

HPV testing within cervical screening programmes requires the use of a rapid, sensitive and practical detection system, capable of automation and high-throughput of clinical specimens. The ability to distinguish between different HPV genotypes and to quantify the amount of HPV DNA present in a clinical sample would be added advantages. The two principal methods currently available for HPV DNA detection in screening applications are Hybrid Capture Assay II (HCAII) and polymerase chain reaction (PCR).

1.12.1 Hybrid capture assay

The first generation tube-based Hybrid Capture Assay has been used in epidemiological studies (Schiffman *et al.*, 1995, Cox *et al.*, 1995) and now a second-generation microplate-based test (HCAII) is commercially available for HPV DNA detection in cervical samples (Lörincz, 1996). This is a molecular solution hybridisation assay in a microplate format for the detection of eighteen HPV genotypes including both high-risk and low-risk genotypes. It is more sensitive than the previous tube-based test (Poljak *et al.*, 1999). Some workers have adapted the signal strength to give a quantitative result reflecting viral load (Clavel *et al.*, 1998), but a correlation between signal strength and grade of cervical lesions has not always been found (Sun *et al.*, 1995; Recio *et al.*, 1998). Some studies have shown the Hybrid Capture technology to be as accurate and sensitive as some PCR assays using PCR

product detection by gel electrophoresis (Farthing *et al.*, 1994; Sun *et al.*, 1995). Others have found it to be less sensitive than consensus, nested or type-specific PCR methods (Cavuslu *et al.*, 1996; Cope *et al.*, 1997).

1.12.2 Conventional PCR

Several PCR methods have been developed to detect a broad spectrum of mucosotropic HPV types, in exfoliated cervical cells and biopsy material, using general or consensus primers to amplify target sequences from either the conserved E1 or L1 open reading frames of the HPV genome. The two most commonly used L1 primer systems are the MY09/11 degenerate primers (Manos *et al.*, 1989) and the GP5+/6+ consensus primers (de Roda Husman *et al.*, 1995a) that amplify a broad spectrum of HPV genotypes, but with variability of detection sensitivity among specific HPV types. Primers derived from conserved regions of the E1 gene include consensus primers GP1 and GP2 that amplify a 444bp target sequence (van den Brule *et al.*, 1990), consensus primers CPI and CPII that amplify a 188bp target sequence (Smits *et al.*, 1992) and IU and IWDO primers that contain inosine residues at certain base positions to increase primer thermal stability (Gregoire *et al.*, 1989). It is impossible to differentiate between different HPV types by agarose gel electrophoresis of consensus PCR amplicons since the amplified HPV products are all very similar in size.

1.12.2.1 PCR primer sets based on L1

1. GP5+/6+

GP5/6 primers were originally designed to detect a broad spectrum of HPV genotypes by allowing mismatch acceptance between two primers and the target DNA during PCR by using conditions of reduced stringency (Snijders *et al.*, 1990). PCR sensitivity of GP5 and GP6 primers (van den Brule *et al.*, 1990; Snijders *et al.*, 1990; van den Brule *et al.*, 1992; Remmink *et al.*, 1995; Kjaer *et al.*, 1996) could be increased 10 to 100 fold by elongation at the 3' end, thus producing GP5+ and GP6+ primers (de Roda Husman *et al.*, 1995a; Jacobs *et al.*, 1995) which amplify a 150bp sequence of the L1 ORF. GP5+/6+ primers were designed to have highest sensitivity for the most common HR-HPV genotype, HPV 16, as

fewest mismatches occur between these primers and the HPV16 L1 ORF target sequence. More mismatches occur between the GP5+/6+ primers and other HPV genotypes so these may be amplified with reduced efficiency.

2. MY09/11

Degenerate MY09/11 primers have been synthesised with nucleotide differences at several positions to make them complementary to the target DNA. Thus MY09/11 primers are a mixture of 25 individual primer sequences that will amplify a 450bp region of the L1 open reading frame.

3. PGMY09/11

The MY09/11 primers have been redesigned to improve the sensitivity of amplification across the range of genotypes. To avoid using degenerate bases, multiple primer sequences were created for the same primer binding region of the L1 gene. These were then combined into an upstream pool of 5 oligonucleotides (PGMY09) and a downstream pool of 13 oligonucleotides (PGMY11). The PGMY09/11 primers are significantly more sensitive than the MY09/11 primers and increased detection of multiple HPV infections from 33.8% to 40% (Gravitt *et al.*, 2000).

The PGMY09/11 oligonucleotide pools have also been adapted for a reverse line blot assay for the simultaneous detection and identification of HPV genotypes (Gravitt *et al.*, 1998). The added advantage of this assay is that it allows discrimination between multiple HPV types in a single hybridisation test. Twenty-seven HPV probe mixes are immobilised onto a nylon strip along with two probe controls and two β -globin cellular controls.

4. “Shorty” primers

For the sensitive detection of high-risk HPV infection only, new primers have been designed from the PGMY system specifically for the detection of high-risk HPVs. These ‘shorty’ primers amplify a 170bp region of the L1 gene and have at least an equivalent, if not higher, level of detection than PGMY primers for thirteen different high-risk types

(Kornegay *et al.*, 2000). Short PCR products also decrease the potential for missing a positive infection due to target DNA degradation in clinical specimens, including DNA recovered from archived material such as paraffin blocks.

5. SPF1/F2

In 1998, a novel broad-spectrum primer set was developed to amplify a 65bp region of the L1 gene. These primers have been shown to be more sensitive than both GP5+/6+ and degenerate MY09/11 primers (Kleter *et al.*, 1998). This short product fragment PCR (SPF-PCR) assay will detect at least 43 different HPV genotypes. Furthermore, a SPF-PCR hybridisation line probe assay (LiPA) was developed using these primers for the simultaneous detection and identification of 16 different HPV types (Melchers *et al.*, 1999). This assay was further developed for genotyping of 25 different HPV types (Kleter *et al.*, 1999).

1.12.2.2 Primer Sets Based on E6/E7

There is some debate about the optimal location of primer binding sites within the genome for HPV DNA amplification. E6 and E7 are always retained following integration but have a lower level of sequence homology throughout the range of genital HPVs, thus E6/E7 primers have detected a limited number of HPV types in some studies (van den Brule *et al.*, 1993). A higher rate of HR-HPV detection was found when E6 primers were compared to L1 in some studies (Lungu *et al.*, 1995, Adams *et al.*, 1996) but a comparison of five HPV primer pairs showed that a combination of two primer sets was essential for identification of all HR-HPV types (Harnish *et al.*, 1999).

1.12.3 Real-time PCR

The commercial LightCycler™ (Idaho Technology Inc., supplied by Biogene Ltd., Kimbolton, Cambridgeshire) combines rapid PCR technology and sophisticated computer software required for programming and product analysis. This allows DNA amplification and analysis of results simultaneously, thus producing a 'real-time PCR' system. Rather than using a metal block thermocycler as used in conventional PCR methods, LightCycler technology utilises circulating air as a medium. In addition, amplification reactions are performed in sealed capillaries made of borosilicate glass, which have a high surface-to-volume ratio. These improvements allow rapid temperature control (maximum ramp rate of 20°C/sec) and hence a significant reduction in testing time (typically 40 cycles in 25 minutes). Commercially prepared master mixes containing reaction buffer, Mg^{2+} , dNTPs and Taq are available to allow fast pre-PCR processing. A fluorescent dye called SybrGreen I™ is added to PCR reactions which binds to the minor groove of double-stranded DNA and emits light on excitation (Wittwer *et al.*, 1997a). This is detected by a microlens at the end of each capillary which bounces the light from a blue light-emitting diode into the volume of liquid, and a photodiode which detects the fluorescence signal generated. Thus, as PCR product accumulates, high precision optics are used to monitor the increase in fluorescence which can be followed on the computer screen.

Because the melting temperature of DNA is dependent on sequence, length and GC content, PCR products can be distinguished by their melting curves. As the temperature increases, dsDNA becomes denatured and SybrGreen molecules become unbound resulting in a decrease in fluorescence. Sudden drops in fluorescence represent the melting of a PCR product and therefore individual products are identified by differences in melting temperature (T_m). The determination of melting curves can be performed on each sample after amplification without opening the reaction vessels, thus reducing the risk of contamination.

A single-tube, real time, nested PCR system has recently been developed to detect HPV DNA using both MY09/11 and GP5+/6+ primers in the same reaction. The sensitivity and specificity is comparable to both PCRs performed separately on a conventional block thermocycler (Strauss *et al.*, 2000).

1.12.4 PCR–ELISA

To make PCR more suitable for routine use, the GP5+/6+ primer system was developed into a rapid, non-radioactive, PCR–enzyme linked immunosorbent assay (PCR–ELISA) for the detection of HPV 16 and 18 DNA. This method is based on GP5+/6+ PCR using one biotinylated primer followed by detection of PCR products in a microwell plate format using DIG-labelled oligoprobes. Due to the additional hybridisation stage this method is more sensitive and specific detecting down to 10–100 HPV copies (Jacobs *et al.*, 1996). This technique was further expanded for group-specific detection of 14 high- and 6 low-risk HPV genotypes (Jacobs *et al.*, 1997) with a sensitivity of 10–200 copies depending on the genotype. A clinical sensitivity of 90% for the detection of CIN II/III lesions has been demonstrated using this system (Nindl *et al.*, 1999). Furthermore, a quantitative PCR–ELISA has been developed to measure the amount of HPV16 DNA per genome equivalent in cervical scrapings. This approach is based on a combined competitive PCR for both HPV16 using GP5+/6+ and β -globin DNA (Jacobs *et al.*, 1999).

Similar PCR–ELISA methods have been developed for the detection of high-risk types using amplification of the E6 ORF followed by detection using fluorescein labelled probes (Lungu *et al.*, 1995). Commercially available systems, such as the SHARP Signal System (Solution Hybridisation Assay for PCR products) and the Boehringer Mannheim PCR–ELISA kit have also been investigated (Terry *et al.*, 1994; Poljak and Seme, 1996).

1.13 Which HPV Test to Use?

In any clinical virology laboratory, there is differentiation between screening and diagnostic assays. Therefore, it is possible that PCR and HCAII may have different uses in particular clinical situations. PCR based tests for HPV DNA detection are highly sensitive and can detect low levels of virus which may be transient and not of clinical significance. Therefore in primary screening, a non-amplification test such as HCAII may be more suitable since highly sensitive HPV detection tests raise the problem of clinical interpretation of low level infections. For clinical management it may not also be necessary to detect the broad range of HPV types, but only detect the HR-HPV infections. Due to the high sensitivity and type specificity achievable with PCR-based tests, they may be useful in ascertaining that no HPV is present when considering extending the screening interval for women with normal cytology. Additionally, PCR assays using consensus primers may be useful in epidemiological studies when it is necessary to detect very low levels of virus so the natural history of HPV can be studied.

Nevertheless, both general PCR and HCA II have important limitations. Both are fairly labour intensive, expensive and neither can differentiate (without follow-up tests) between individual HPV types or detect infection with multiple HPV types. After PCR, follow-up investigations that will identify individual HPV types include sequencing (van den Brule *et al.*, 1992), type-specific probes (de Roda Husman *et al.*, 1994; Jacobs *et al.*, 1995) or restriction fragment analysis (RFLP) of PCR products (Adams *et al.*, 1996).

The major hurdle facing the introduction of HPV testing into existing cervical screening programmes, regardless of the HPV testing method of choice, is the issue of cost. So far, little information is available. Most studies assessing the economic implications of the introduction of HPV testing have used mathematical models to assess the potential resource savings by a reduction in the number of repeat Pap smears and referrals for colposcopy or histological follow-up (van Ballegooijen *et al.*, 1997; Brown and Garber, 1999; Goldie *et*

al., 2001). They do not however address the actual cost savings in terms of the number of cancers prevented or the numbers of life years gained by reduced mortality. Cuzick and Sasieni (1997) estimated that if the introduction of HPV testing would allow safe extension of the cervical screening interval from 3 to 5 years, then a saving of approximately £30 million per year would be possible. To confirm this, large longitudinal screening studies are required which must also include assessment of the financial costs of combined liquid-based cytology and HPV testing. In France, one such study has been performed to compare the cost effectiveness of LBC and HPV testing with that of conventional Pap smears for cervical cancer screening (Cochand-Priollet *et al.*, 2001). In the UK, similar trials are underway which have health economics cost analysis incorporated in the study design.

1.14 Summary

Despite the close association between HR-HPV infection and the development of cervical cancer, there is some reluctance to use HPV testing as a primary tool for cervical cancer screening due to the occurrence of a few cases of HPV negative cervical cancer. There is some evidence that HPV negative cancers are found in older women and are associated with a poorer prognosis (Walboomers and Meijer, 1997). In contrast, there is also much controversy about whether such cases represent a true HPV-negative cervical carcinoma subset or whether false HPV negative results have been generated reflecting either disruption of HPV during integration, failure to detect a new HPV type or very low levels of viral DNA. In addition, HPV testing has lower specificity than cytology for high-grade CIN, especially in younger women.

On the other hand, a strong clinical case can be made for the application of HPV DNA testing for triage of women with B/L smears by providing a more objective test result that will improve identification of premalignant cervical lesions. This has the potential to increase the sensitivity and specificity of the NHSCSP whilst reducing the costs by rationalising the management for individual women via reduced repeat smears and colposcopy clinic visits.

1.15 Aim and Topic of This Thesis

This work in this thesis was performed to investigate the role of HPV testing in the NHSCSP for improved detection of cervical neoplasia, with particular emphasis on the suitability of HPV testing methods for use in a diagnostic laboratory setting.

With the advent of liquid-based cytology, the suitability of preservative fluids for DNA extraction was assessed, in both split-sample and direct-to-vial studies, since this allows both cytomorphological assessment and HPV tests to be performed from the same cervical sample. To allow high throughput HPV testing of clinical LBC specimens, an automated DNA extraction protocol was developed using a QIAGEN BioRobot 9604®.

The introduction of HPV testing must improve the sensitivity, specificity and predictive value of the current methods. Two of the most well established techniques, Hybrid Capture II and GP5+/6+ PCR, were used extensively for HPV DNA detection. Since, however, neither of these methods can distinguish between individual HPV genotypes, novel approaches for HPV detection and identification were also evaluated. To improve sample turn-around within the laboratory, a real-time PCR assay was developed for rapid HPV DNA detection and further identification of specific HPV types. Genotyping was also performed by DNA sequence analysis and a reverse line blot assay (under development) to determine the exact HPV types infecting the cervix and to distinguish between persistent HPV infection or re-infection with different HPVs. In an attempt to determine whether HPV testing would be most effectively targeted within the CSP for HPV screening or diagnosis, the above tests were selectively performed on cervical specimens collected from specific patient populations including women with previous borderline cytological abnormalities, women attending for routine cervical screening and immunocompromised women.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1. Preparation of cytology slides using ThinPrep® 2000

Technical staff at the University of Edinburgh Pathology Department (UEPD) made ThinPrep® smears using ThinPrep® 2000 technology. Sample vials were placed one at a time into the ThinPrep® machine. Mucous, blood or non-diagnostic debris was first broken up by gentle dispersion and then the sample was thoroughly mixed. A series of negative pressure pulses were generated that allowed the fluid to be drawn through a filter. The appropriate amount of diagnostic cellular material was then transferred to a defined screening area on a glass slide using mechanical positioning and positive air pressure. The slide, which contains a thin even layer of cells that is virtually free of obscuring artefacts, was fixed using CellFyx™ solution (Cytoc Corporation) before Pap staining was performed using the standard method. Occasionally it was necessary to prepare a second slide in order to obtain a satisfactory thin layer preparation.

2.2. Automated Screening of Cytology Slides

For ethical purposes, a permanent record was made of some cytology slides before destruction. PAPNET uses neural network computer technology to analyse each of the cells in a Pap smear and make a permanent record of the 128 most suspicious cells. Slides were sent to PAPNET NSI, (NSI Europe B.V., Zekeringstraat, Amsterdam) for this purpose.

2.3. Cervical Cytology Reporting

Trained Cytotechnologists at the UEPD screened all the conventional Pap cervical smears. UEPD serves the South-East Scotland population of 250,000 women in the screening age group (18–60 years) which generates an annual workload of 85,000 smears. Cytological appearances were classified as borderline, mild, moderate or severe dyskaryosis according to the BSCC's terminology and guidelines for adequacy of cervical smears (Evans *et al.*, 1986). LBC smears were screened by particular staff members trained in this method.

2.4. Management of Cervical Disease

Follow-up of cytological abnormalities was performed at the Royal Infirmary of Edinburgh Colposcopy Clinic. Patients with histological evidence of CIN of any grade were treated using either cold coagulation or, more recently, loop diathermy in the conventional manner performing a LLETZ which allows histological confirmation of the lesion.

2.5. DNA Extraction from Clinical Specimens

2.5.1. Guanidinium isothiocyanate/ silica method

After soaking the archival smears in xylene for 2-7 days to remove the coverslips, cells were scraped from each slide using a sterile scalpel blade and transferred to an eppendorf tube containing 1ml of xylene. DNA extraction was performed using a modification of the guanidinium isothiocyanate (GTC)/silica method of Boom *et al.*, (1990). After centrifugation, the cell pellets were resuspended in 900µl GTC-containing lysis buffer (Appendix 1), mixed thoroughly and incubated at room temperature for 2.5 hours. 40µl of sterile silica beads in 0.1M HCl were added and incubated for one hour at room temperature with occasional mixing. The silica beads and bound DNA were centrifuged at 5000 rpm for 1 minute, washed twice with 500µl GTC-containing wash buffer and once with 70% ethanol. After the pellets were air-dried at room temperature for 30 minutes, the DNA was eluted by addition

of 250µl TE buffer, pH8.0 at 58°C. DNA was then precipitated using 1ml 96% ethanol/50µl 3M sodium acetate, pH5.2. Finally, the cell pellets were washed in 70% ethanol, air dried and then resuspended in 120µl sterile water.

2.5.2. QIAamp® DNA mini kit

QIAamp® extraction procedures from QIAGEN Ltd. (West Sussex, UK) use a modification of the method of Boom *et al.*, (1990) to purify DNA. After specimen processing, cell pellets were resuspended in 200µl TE buffer, pH7.2 for DNA extraction using the QIAamp® DNA Mini Kit which is designed for purification of DNA from human tissue samples such as muscle, heart, liver and brain. Due to the relatively high mucous content of LBC samples and the large volume of sample remaining after the thin layer smear preparation had been made, it was found that the QIAGEN protocol for body fluids was unsuitable for efficient extraction. Thus, the tissue protocol, which has an improved lysis stage, was necessary.

180µl of primary lysis buffer (ATL) and 20µl of proteinase K were added to each sample which was incubated at 55°C for at least one hour in a water bath (particular mucous-rich specimens required longer incubation times of up to 2 hours). 200µl of secondary lysis buffer (AL) was then added and incubated at 70°C for 10 minutes. After the addition of 210µl ethanol, the sample was transferred to a QIAamp® spin column in a 2ml collection tube and centrifuged at 8000 rpm for one minute. The filtrate was discarded before addition of wash buffer (AW1) then centrifugation at 8000 rpm for 1 minute. The filtrate was again discarded before addition of 500µl wash buffer (AW2). Following centrifugation at 13000 rpm for 3 minutes, the DNA was eluted twice using 200µl Buffer AE. Both eluates were pooled to give a final elution volume of 400µl.

After showing the suitability of this extraction method for cervical specimens, cell pellets were then stored directly in ATL buffer and frozen at either -20°C or -70°C.

2.5.3. QIAVac® 24

The QIAGEN QIAVac® 24 system was designed for convenient vacuum processing of up to 24 spin columns in parallel. Samples and wash buffers were drawn through the column membranes under vacuum rather than by centrifugal force. This reduced the hands-on-time involved during the QIAamp® extraction protocol thus increasing the speed of the extraction process. Some QIAamp® spin columns were processed using VacConnectors with VacValves. The VacValves were inserted directly into the luer extensions of the QIAVac® 24 manifold. VacConnectors (disposable connectors that fit between the QIAamp® spin columns and the VacValves) were used to prevent direct contact of the spin columns and VacValves thus avoiding any cross-contamination. They were discarded after a single use. Vacuum pressure was generated using a water-pump thus drawing liquid through the silica membranes. If the sample flow rates differed significantly between samples then the VacValves could be opened or closed to ensure a consistent vacuum.

2.6 Hybrid Capture Assay II (HCAII)

HCAII (Digene Diagnostics Ltd.) is a molecular solution hybridisation assay for the qualitative detection of thirteen high-risk HPVs and 5 low-risk genotypes using two separate ssRNA probe cocktails to distinguish the oncogenic and non-oncogenic types. In the majority of studies, this assay was performed using the HR-HPV probe cocktail only for the detection of HR-HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68 that are associated with cervical neoplasia and cancer. The low-risk probe will detect HPV 6, 11, 42, 43 and 44.

After specimen processing, DNA was denatured using a denaturation reagent (DNR) containing sodium hydroxide and purple indicator dye which was added in half volumes as compared to the specimen starting volume (e.g. 500µl per 1ml sample). After mixing, DNA was denatured in a waterbath at 65°C for one hour. 25µl of RNA probe cocktail was then added to the samples, resulting in a colour change from purple to yellow. The samples were shaken on a rotary shaker at 1100 rpm for 5

minutes and then re-incubated at 65°C for another hour to allow hybridisation to occur. After a 5 minute cooling period, the 100µl sample volume was transferred to microtitre capture plate wells that were coated with anti- RNA:DNA hybrid antibodies. During a one hour hybridisation period (with shaking), hybrids were immobilised onto the solid surface. After blotting, detection reagent containing alkaline phosphatase conjugated antibodies was then added and the plate incubated at room temperature for 30 minutes. The plate was then washed manually six times using Digene wash buffer and blotted well. A chemiluminescent substrate was then added that was cleaved by the bound alkaline phosphatase producing a light signal that can be measured accurately. Since multiple conjugated antibodies can bind to each hybrid, substantial signal amplification occurred. After a 15 minute incubation in the dark, the emission of light was measured as relative light units (RLUs) using a DML 2000 luminometer connected to a computer. The RLU value obtained was proportional to the amount of target DNA present. Samples with a RLU/cut-off value >1 indicated a positive result for any of the HPV types contained in the probe pool.

Positive and negative control material was included in every run. Each control was run in triplicate and the assay cut-off value was calculated from the positive control results obtained. The negative control contains carrier DNA in Digene Specimen Transport Medium (STM). The positive control contained 1pg/ml HPV 16 DNA in STM.

It should be noted that the first-generation, tube based HCA (HCA I), was used at the beginning of one study (Chapter 5) and was replaced by the second-generation test (HCA II) as soon as it became available. The HCA I methodology is not discussed here as samples were retested with HCAII, and HCAI only detected nine HR-HPV types which were also detectable by HCAII. In addition, the study using DNA extracted from archival smears was performed using a protocol adapted for 100µl amounts following manufacturers' instructions (Chapter 4).

2.7 Sample Conversion for HCAII

Before HCA II testing, PreservCyt® specimens required initial processing using a Digene Sample Conversion Kit. After vortex mixing, a 4ml volume of PreservCyt® fluid was removed from the specimen vial into a 20ml sterile universal. 0.4 ml of Digene Sample Conversion Buffer was added to each sample, mixed then centrifuged at 3660rpm for 15 minutes. A stock solution of STM/DNR mix was made by adding 120µl STM and 60µl DNR per sample to be tested. After centrifugation, the supernatant was removed from each sample and the cell pellet resuspended in 150µl STM/DNR mix. This volume was then transferred to a sterile, labelled sarstedt vial and denatured at 60°C for 45 minutes. Samples were then stored at -20°C until batch HCAII testing was performed, starting at the hybridisation stage.

2.8 Conventional PCR

The PCR is a molecular amplification technique that utilises a heat-resistant DNA polymerase from *Thermus aquaticus* to extend primers chosen to flank a target region of DNA. Multiple heating and cooling steps result in alternating cycles of DNA synthesis and denaturation leading to an exponential accumulation of the specific PCR product defined at its ends by the positions of the primers.

2.8.1 GP5+/6+ PCR

The general primers GP5+ and GP6+ (Table 2.1) were used to amplify a 150bp region of the L1 gene. These consensus primers detect at least 27 different mucosotropic HPV genotypes, including both high- and low-risk genotypes, simultaneously by allowing for base mismatching during primer binding at a low annealing temperature of 40°C. A 150bp region of the major capsid protein gene can be amplified using this pair of primers from each of HPV 6, 11, 13, 16, 18, 30, 31, 32, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 55, 56, 57, 58, 59, 61 and 66 (de Roda Husman *et al.*, 1995a).

PCR amplification was performed in 50µl PCR solution containing 25pmols of each primer, 200µM of each deoxynucleoside triphosphate, 1× reaction buffer, 3.5mM MgCl₂, 1U Taq polymerase (Life Technologies Ltd, Paisley, Scotland) and 10µl extracted DNA. The mixture was overlaid with oil and then subjected to 40 cycles of amplification (1 minute denaturation at 94°C, 2 minutes primer annealing at 40°C, 1.5 minutes DNA synthesis at 72°C). The first cycle was preceded by 4 minutes of denaturation at 94°C and the last cycle was extended by 4 minutes at 72°C. At least one positive and negative control was included in each PCR run.

| Primer Name | Sequence (5' to 3') | Product size |
|-------------------------|---------------------------|--------------|
| GP5+ | TTTGTTACTGTGGTAGATACTAC | 150bp |
| GP6+ | GAAAAATAAACTGTAAATCATATTC | |
| β-globin forward primer | CAACTTCATCCACG TTCACC | 209bp |
| β-globin reverse primer | ACACA ACTGTGTTC ACTAGC | |

Table 2.1 Sequences of PCR primers

2.8.2 β-globin PCR

β-globin primers (Table 2.1) were used for amplification of a 209bp region of this cellular gene (Saiki *et al.*, 1985) as a positive control to confirm that cellular DNA was present and could be amplified by PCR.

PCR amplification was performed in 50µl PCR solution containing 50pmol of each primer, 200µM each deoxynucleoside triphosphate, 1× reaction buffer, 1.5mM MgCl₂, 1U Taq polymerase (Life Technologies) and 10µl extracted DNA. The mixture was overlaid with oil and then subjected to 40 cycles of amplification (1 minute denaturation at 95°C, 2 minutes primer annealing at 55°C, 1.5 minutes DNA

synthesis at 72°C). The first cycle was preceded by 4 minutes of denaturation at 95°C and the last cycle was extended by 4 minutes at 72°C. At least one positive and negative control was included in each PCR run.

2.8.3. HotStart PCR

To minimise the amount of non-specific PCR amplification and reduce primer-dimer artefacts, TaqStart™ Antibody (Sigma-Aldrich Co. Ltd., Dorset, England) was added to most PCR reactions. This is a neutralising monoclonal antibody that binds to Taq polymerase thus preventing its activity in the PCR reaction mix. When the temperature is raised above 90°C during the first high temperature step in the PCR process, the enzyme-antibody complex dissociates. This results in the restored activity of the Taq polymerase enzyme while the TaqStart™ Antibody is rendered non-functional by heat denaturation.

For each PCR reaction mix, a working solution of TaqStart™ antibody was made. For 10 PCR amplifications, 4.4µl TaqStart™ Antibody was added to 17.6µl of dilution buffer (provided by the manufacturer). 4.4µl of Taq polymerase (5units/µl) was then added to this diluted TaqStart™ Antibody and incubated at room temperature for 10 minutes to allow binding of the antibody to the enzyme. 2.4µl of this mix was then added per PCR reaction.

2.8.4 PCR contamination precautions

All PCR reactions were performed under conditions designed to minimise sample contamination. DNA isolation, preparation of PCR reactions, sample addition and analysis of PCR products was conducted in four separate areas. Dedicated pipettes with sterile filter disposable tips were used at each stage. All reagents and amplification tubes used in each run were prepared in the PCR 'clean room'. DNA samples were added to the PCR mix in a separate sample loading room. PCR was performed using either Hybaid Omni-e or Hybaid Omnigene thermocyclers followed by PCR product analysis in an additional area used only for gel electrophoresis.

2.9 PCR Controls

2.9.1. Cultured cell lines

Cells from the human cervical cancer cell lines SiHa and HeLa were obtained from laboratory stocks frozen in liquid nitrogen. SiHa and HeLa cells contain respectively 1–2 copies of HPV16 DNA (El Awady *et al.*, 1987) and 30–40 copies of HPV 18 per cell (Arends *et al.*, 1991).

These cell lines were cultured in Eagles Balance Salt Solution with 10% foetal bovine serum (Appendix 1) until monolayers became confluent (in approximately 4–5 days). The monolayers were then washed twice with 4–5ml of Dulbeccos solution to remove serum and then 0.8ml 2.5% trypsin in 10ml Dulbeccos solution was added. After approximately 2 minutes, the trypsin was removed and each flask was incubated at 36°C until the cells could be shaken from the glass (< 2 minutes). Following resuspension in 5ml growth medium, the cells were counted using an 'improved Neubauer' counting chamber. 100µl of cells was added to 900µl trypan blue stain and a drop of this mix was allowed to fill an area on the counting chamber under a coverslip. The number of viable cells/ml was calculated. Cells were either stored in 1ml volumes at –20°C (with the cell count noted) or used to reseed another flask at 0.6m cells/flask. DNA was extracted from 200µl of cells with a known cell count/ml using the QIAamp® DNA Mini Kit protocol in accordance with the manufacturer's tissue protocol. Extracted DNA from SiHa and HeLa cells was used as positive PCR control material.

2.9.2. Cloning of HPV 16 and HPV 18 L1 DNA

DNA was extracted from four separate aliquots of both SiHa and HeLa cells using the QIAGEN Tissue extraction protocol. GP5+/6+ consensus primers were used to amplify the L1 region. The integrity of the PCR product was analysed by agarose gel electrophoresis and an estimate of the PCR product concentration was made. The L1 region was cloned into the pGEM®-T Easy Vector using the pGEM®-T Easy Vector System (Promega UK, Southampton, England).

The high copy number pGEM®-T Easy Vector (3018bp) contains T7 and SP6 RNA Polymerase promoters flanking a multiple cloning site (MCS) within the α -peptide coding region of the enzyme β -galactosidase (Lac Z gene). Successful cloning into overhanging T ends interrupts the coding sequence of β -galactosidase allowing recombinant clones to be directly identified by colour screening on indicator plates. The pGEM®-T Easy Vector contains multiple restriction sites within the MCS including recognition sites for the restriction enzymes *EcoR* I, *BstZ* I and *Not* I, to allow for the release of the insert by digestion with a single restriction enzyme.

Depending on the concentration of PCR product formed, either 2 μ l (for strong PCR product) or 4 μ l (for weak PCR product) of PCR product was added to each 10 μ l ligation reaction. Each ligation reaction also contained 10 \times buffer, 1 μ l pGEM®-T Easy Vector (commercially prepared by cutting Promegas pGEM®-5zf(+) Vector with *EcoR* V and adding a 3'-terminal thymidine to both ends), 1 μ l T4 DNA ligase (3 Weiss units/ μ l) and deionised water. The mixtures were incubated at 4°C overnight. One positive control, containing a control insert DNA (to allow assessment of ligation efficiency), and one negative control (comprising vector with no insert), were included following the manufacturer's instructions. The negative control allowed determination of the number of background blue colonies resulting from non-tailed or undigested pGEM®-T Easy vector alone.

Transformation experiments were performed at the Department of Veterinary Medicine, Summerhall, University of Edinburgh using JM109 High Efficiency Competent Cells (kindly provided by Dr Bob Daziel). These bacterial cells were compatible with blue/white screening and standard ampicillin selection. Following brief centrifugation, 2 μ l of each ligation reaction was added to a 1.5ml eppendorf tube on ice. Another tube with 0.1ng uncut plasmid for determination of the transformation efficiency of competent cells was also prepared. JM109 cells were then removed from -70°C storage and thawed on ice for approximately 5 minutes. After gentle mixing, 50 μ l of cells was added to each ligation reaction tube and 100 μ l

of cells was added to the transformation efficiency control tube. After mixing by inversion, the tubes were cooled on ice for 20 minutes. Cells were then heat-shocked by placing the tubes in a water bath set at exactly 42°C for 45–50 seconds. The tubes were then immediately placed back on the ice for 2 minutes. 950µl SOC medium (Appendix 1) at room temperature was added to the ligation reaction tubes and 900µl SOC medium was added to the transformation efficiency control tube. All tubes were then incubated at 37°C (with shaking) for one hour. The transformation efficiency of the competent cells was checked by transforming them with an uncut plasmid according to manufacturers' instructions. 100µl of each transformation culture was plated onto duplicate LB/ ampicillin/ IPTG/ X-gal plates. A 1:10 dilution with SOC medium was made for the transformation control as recommended. All plates were incubated overnight at 37°C. Clones that contained PCR product inserts produced white colonies. Colonies were picked and grown overnight at 37°C in LB medium (Appendix 1). Plasmid DNA was then isolated using QIAGEN QIAprep® Plasmid Miniprep procedure according to manufacturers' instructions. In summary, the pelleted bacterial cells were resuspended in 250µl of Buffer P1 (provided by the manufacturer) to which 250µl of Buffer P2 (provided by the manufacturer) was added. After mixing, 350µl of Buffer N3 (provided by the manufacturer) was then added and samples were centrifuged for 10 minutes. The supernatant from each sample was transferred to a 2ml collection tube. After brief centrifugation (30–60 seconds), the flow-through was discarded and endonucleases were removed by a wash with 0.5ml of Buffer PB (provided by the manufacturer). This was followed by another brief centrifugation step and the flow-through was discarded. 0.75ml of Buffer PE (provided by the manufacturer) was then added to wash the membrane and remove any salts present. After centrifugation, the flow-through was again discarded and centrifugation performed for an additional 1 minute to remove residual wash buffer. DNA was eluted into a fresh 1.5ml tube by addition of 50µl of sterile water and centrifugation for 1 minute. The plasmid DNA was then treated with restriction enzyme *EcoRI* (provided by Dr. Daziel).

2.9.3 Plasmid preparation

The DNAs of HPVs 16, 18, 45, 6 and 11 isolated from clinical material, were kindly provided by Prof. Dr. E-M de Villiers (Referenzzentrum fuer humanpathogene Papillomviren, Heidelberg, Germany). HPV 6 was provided cloned in vector pUC19; HPVs 11, 16, 18 in pBR322 and HPV 45 in pGEM4. HPV 33 was cloned in plink322 obtained from Dr G. Orth (Institut Pasteur, Paris, France) and HPV 31 was cloned in pT713 and was obtained from Dr A. Lorincz (Digene Diagnostics, Gaithersburg, MD, USA).

Transformation was performed using Library Efficiency DH5 α TM Competent Cells (Life Technologies, Paisley, Scotland). Plasmid-containing colonies were selected using L-Amp plates then cultured in L-broth containing ampicillin. Plasmid DNA was isolated from this bacterial cell culture using Hybaid Recovery Quick Mini Spin Kit (Hybaid Ltd., Middlesex, England) following the manufacturers' instructions. This procedure utilises a modified alkaline/SDS method and spin column technology to prepare a pure lysate. After centrifugation of the bacterial cells, the culture medium was carefully removed and 210 μ l cell lysis solution was added and mixed by inversion. The samples were then incubated at room temperature for 5 minutes. 280 μ l of neutralising solution was added, the samples mixed by inversion and then centrifuged at 13000 rpm for 10 minutes. The lysate was then added to a spin column and centrifuged at 13000 rpm for 1 minute to bind the plasmid DNA to the silica membrane. After washing, using 700 μ l wash solution, the plasmid DNA was eluted into a 1.5ml eppendorf tube using 75 μ l sterile water added directly to the centre of the silica matrix. A final centrifugation step was performed at 13000 rpm for 2 minutes.

Extracted DNA was digested with restriction enzymes and electrophoresed in 1% agarose to check the identity of DNA. Finally, GeneQuant II spectrophotometric analysis (Amersham Pharmacia Biotech, St. Albans, Herts, England) was performed to measure the DNA concentration. This work was carried out in the Department of Pathology, University of Edinburgh under the supervision of Mr. Robert Morris.

2.10 Reverse Line Blot Assay (RLBA)

Biotinylated PGMY09/PGMY11 primers (provided by Roche Molecular Systems) were used to amplify a 450bp fragment of the L1 ORF from a broad range of genital HPV types and biotinylated BGH20/BPC04 primers were used to simultaneously amplify a 268bp fragment of the human β -globin gene (Roche Molecular Systems, Alameda, CA). Each 100 μ l PCR solution contained 1.05 μ M of PGMY09/11 primers, 0.05 μ M of BGH20/BPC04 primers, 200 μ M of dATP, dCTP and dGTP, 600 μ M of dUTP, 1 \times reaction buffer II, 4mM MgCl₂, 7.5U AmpliTaq Gold and 5 μ l extracted DNA. dUTP was substituted for dTTP so that potential laboratory carryover contamination with HPV or β -globin PCR products was eliminated with the incorporation of uracil-N-glycosylase (UNG). The mixture was then subjected to 40 cycles of amplification (30 seconds at 95°C, 1 minute at 55°C, 1 minute at 72°C). The first cycle was preceded by 9 minutes of denaturation at 95°C. After the last cycle samples were held at 4°C until required for the next step.

After amplification, the PCR product was denatured by addition of 100 μ l of Amplicor Denaturation Solution (provided by manufacturer) to each PCR reaction tube and incubated at room temperature for 15 minutes. 75 μ l of denatured PCR product was then added to a well in a tray containing a single HPV genotyping strip in 3ml of pre-warmed hybridisation solution (at 53°C). Strips were placed on a platform shaker (set at 70 rpm) inside an incubator set at 53°C for 30 minutes.

After removing the hybridisation solution from the wells by aspiration, 3ml of room temperature wash solution was added to each tray well, mixed, and then immediately aspirated to remove any residual hybridisation solution. 3ml of pre-warmed wash solution (at 53°C) was added and shaken for 15 minutes at 53°C. The wash buffer was then removed by aspiration and 3ml of conjugate (streptavidin-horseradish peroxidase) solution was added to each well. The tray was shaken at 50rpm for 30 minutes at room temperature. After removing the conjugate solution from the wells by aspiration, 3ml of room temperature wash solution was added to each well, mixed, and immediately aspirated to remove any residual conjugate solution.

Another 3ml of room temperature wash solution was then added to all wells and the tray shaken for 10 minutes on the platform shaker.

Following aspiration, this was repeated with another 10 minute wash step. After aspiration of the wash solution, 3ml of citrate buffer, made from a 20× stock solution provided by the manufacturer, was added to each strip and incubated for at least 5 minutes. The buffer was removed by aspiration then 3ml of colour development solution added to each well and the tray was shaken at 50rpm for 5 minutes. After aspiration of the colour development solution, the strips were heat sealed in plastic pockets containing a small volume of citrate buffer. The strips were interpreted manually using the HPV Overlay guide provided (Figure 2.1) then stored in the dark at 4°C. Both control lines had to be positive for the test to be considered valid. For interpretation purposes, the intensity of the bands present were graded as +, ++ and +++ representing a weakly positive, positive and strongly positive hybridisation result respectively.

2.11. Agarose Gel Electrophoresis

After amplification, PCR products were analysed by gel electrophoresis using 2% agarose gels stained with ethidium bromide using 1× TAE electrophoresis buffer (Appendix 1). A 1kb DNA molecular weight ladder (Life Technologies) was used to confirm the size of the PCR products generated. Gels were electrophoresed at 70–100 volts for 40 minutes. A photographic record of the gels under UV light was made using Sigma black and white Polaroid film.



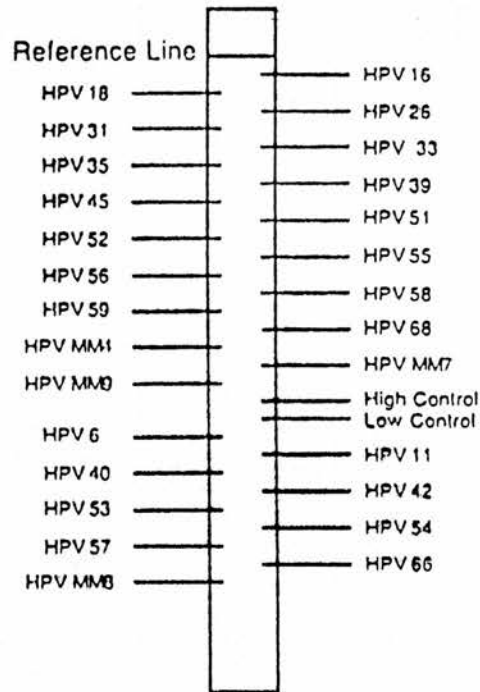


Figure 2.1 Overlay guide for the RLBA showing the HPV genotypes detected on each test strip. 27 HPV typing probes are immobilised on a single nylon strip and two control probes (high positive and low positive) that hybridise to the amplified β -globin control product.

2.12. Purification of PCR Products

Before sequencing was performed, samples showing non-specific amplification were purified using QIAquick® Gel extraction kit (QIAGEN Ltd.) following manufacturer's instructions. Firstly, DNA fragments of the correct size were excised from the 2% agarose gel under UV illumination, using a sterile scalpel blade. The fragments were then treated with 300µl Buffer QG (provided by manufacturer) at 50°C for 10 minutes. This solubilised the agarose gel slice and provided the appropriate conditions for binding of DNA to the silica membrane. After addition of 100µl isopropanol, the sample was transferred to a QIAquick® spin column in a 2ml collection tube and centrifuged for 1 minute at 13,000 rpm. The flow through was discarded and then 0.5ml Buffer QG added to each column before a further centrifugation step. Washing of the membrane was performed using 0.75ml Buffer PE (provided by manufacturer), which was left for 5 minutes before centrifugation. A dry spin of the column was then performed. The DNA was eluted by addition of 30µl of Buffer EB (provided by manufacturer) to the centre of the QIAquick® membrane followed by a final centrifugation step. 10µl of the eluted DNA was used in the PCR amplification reaction. After gel electrophoresis a clean, specific product was observed and a volume of this PCR product was used for sequencing.

2.13. Restriction Enzyme Digests

Restriction enzymes are used to cut DNA at particular recognition sites to generate fragments of a defined length that can be distinguished by agarose gel electrophoresis. The restriction enzymes used were *Bam* *H*I (recognition site 5' G/GATCC 3'), *Eco* *R*I (recognition site 5' G/AATTC 3'), *Bgl* *II* (recognition site 5' A/G°AT*CT 3') and *Hind* *III* (recognition site 5' A/AGCTT 3'). All enzymes were provided by Sigma-Aldrich Co. Ltd. (Poole, Dorset, England). Each reaction contained 11µl of sterile water, 2µl 10× buffer, 2µl enzyme and 5µl DNA and digests were performed in a 37°C water bath for 1–2 hours.

2.14. DNA Sequencing

DNA sequencing was performed by staff at DNASHEF Technologies (Department of Haematology, Royal Infirmary of Edinburgh). Both strands of the PCR product were sequenced using ABI Prism® BigDye™ Terminator Cycle sequencing with Ready Reaction Kits (Applied Biosystems, Perkin Elmer United Kingdom, Warrington, UK). Reaction mixes were prepared by adding 4µl Terminator Ready Reaction Mix (which contains fluorescently labelled A-,C-,G-, and T-Dye Terminators, dNTPs, AmpliTaq DNA polymerase, MgCl₂ and Tris-HCl pH9), 2µl SB Buffer (5×), 1–5µl of PCR product, 1µl primer (GP5+ or GP6+) and 8–12µl deionised water.

The tubes were placed in a thermocycler and subjected to 25 cycles of the following: 96°C for 10 seconds; 50°C for 5 seconds then 60°C for 4 minutes. Samples were held at 4°C until ready to purify. After centrifugation, ethanol/sodium acetate precipitation was performed. For each sequencing reaction, 2µl 3M sodium acetate, pH4.6 and 50µl 95% ethanol were added to a 1.5ml eppendorf tube. The entire volume of extension reaction product was added to this mix. After mixing by inversion, the extension products were left to precipitate at room temperature for 15 minutes. Samples were centrifuged at 14000rpm for 20 minutes and the supernatant was carefully aspirated. The pellets were rinsed in 250µl 70% ethanol, vortexed and then centrifuged at 14000rpm for 5 minutes. The supernatant was again carefully removed by aspiration and the pellets were placed in a heat block at 96°C for 2 minutes until dry. The pellets were resuspended in 4µl of a 5:1 mixture of formamide/blue dextran-EDTA, denatured at 96°C for 2 minutes and placed on ice until ready to use. Electrophoresis of samples was performed on a 4% polyacrylamide gel using the ABI PRISM 377. Data was saved as chromatogram files that could be transported electronically. DNA sequence files were visualised using chromas software package 1.42 (Griffith University, Brisbane, Australia) via internet citation www.technelysium.com.au/chromas.html.

The DNA sequences from both strands were aligned and compared to published HPV sequences in the GenBank database (Los Alamos National Laboratories) using

Basic Local Alignment Search Tool (BLAST) software (Altschul *et al.*, 1990) via the National Centre for Biotechnology Information web site at <http://www.ncbi.nlm.nih.gov/>. If the sequence could not be interpreted, due to more than one peak present at the same position at multiple sites, this was considered to signify a mixed infection with more than one HPV genotype represented by two or more DNA fragments in the PCR solution.

CHAPTER 3

NEW OR MODIFIED METHODS DEVELOPED FOR HIGH-THROUGHPUT HPV ANALYSIS

3.1. Liquid-Based Cytology Specimen Processing for Automated DNA Extraction

After a ThinPrep® smear had been prepared, the total volume of residual PreservCyt® fluid in the sample vial was processed for DNA extraction in an attempt to increase the potential for detecting low-level HPV infections. The fluid was measured in a sterile universal tube with the volume noted and the cells present were pelleted by centrifugation at 3600 rpm for 15 minutes. An additional note was made if the sample was particularly mucoid or bloody in nature. After centrifugation, the supernatant was discarded and the pellet resuspended in 180µl ATL lysis buffer (provided by QIAGEN Ltd.) and transferred to a labelled, sterile sarstedt vial. In addition, some samples were processed using either 10ml, 5ml or 1ml of the residual PreservCyt® fluid. This was done to assess the sensitivity of the automated DNA extraction and PCR systems.

Furthermore, some of the samples that had 10ml only processed were also subjected to treatment with CytoLyt™ (CL) fluid (Cytoc Corporation), a methanol-based, buffered preservative solution designed to lyse red blood cells, prevent protein precipitation, dissolve mucous and preserve morphology of cytology samples. It was designed as transportation medium for non-gynaecological cytology samples such as respiratory and gastrointestinal specimens, prior to processing. After initial centrifugation of the 10ml specimen volume in a sterile universal, the supernatant PreservCyt® fluid was removed and replaced with 10ml of CL solution. After thorough mixing, samples were centrifuged at 3660 rpm for another 10 minutes and the supernatant decanted. The cell pellet was then resuspended in 180µl ATL buffer, ready for DNA extraction.

3.2 Automated DNA Extraction using BioRobot® 9604

The BioRobot® 9604 (QIAGEN Ltd.) is a molecular biology workstation for nucleic acid extraction and purification from clinical samples attached to a PC with dedicated software (Figure 3.1 and Figure 3.2). The BioRobot platform contained a specimen rack area to hold the processed specimen pellets in ATL buffer (12 racks containing eight samples in a 96-well plate format). Four individual probes attached to a robotic arm allowed movement across the platform. Six racks of plugged tips were loaded onto the workstation. These tips were attached to the probes for the delivery of certain reagents, which were loaded into defined slot areas on the BioRobot platform. Plugged tips were used to transfer an 180µl aliquot of the sample from the specimen rack to a plastic 96-well square block containing diluted proteinase K. This block was loaded on an electronically controlled thermostat system which was used to incubate the samples at 56°C for one hour for sample lysis. 200µl of a secondary lysis buffer (AL; provided by the manufacturer) was added to the samples before another incubation step at 70°C for 10 minutes. At this stage, ethanol was added to a separate 96-well square block to which the lysates were added before transfer of the entire volume to the QIAamp® 96 vacuum plate which was located on the vacuum manifold. To prevent contamination, an overlay of wash buffer (AW1; provided by the manufacturer) was added before samples were drawn through the QIAamp® membranes under vacuum pressure driven by a peristaltic pump. During this process DNA was bound to the membrane while residual material was washed away. The membranes were washed with wash buffers AW1 and AW2 (provided by the manufacturer), pumped from individual buffer bottles, and the plate was centrifuged at 6000 ×g for 10 minutes with a micropore sheet attached to dry the membranes before the elution step was performed. DNA was eluted into plastic collection microtubes using 200µl AE buffer. Two separate elution steps were performed to give a final elution volume of 400µl. The tubes were then sealed with strip caps, labelled and stored at -70°C until PCR testing.

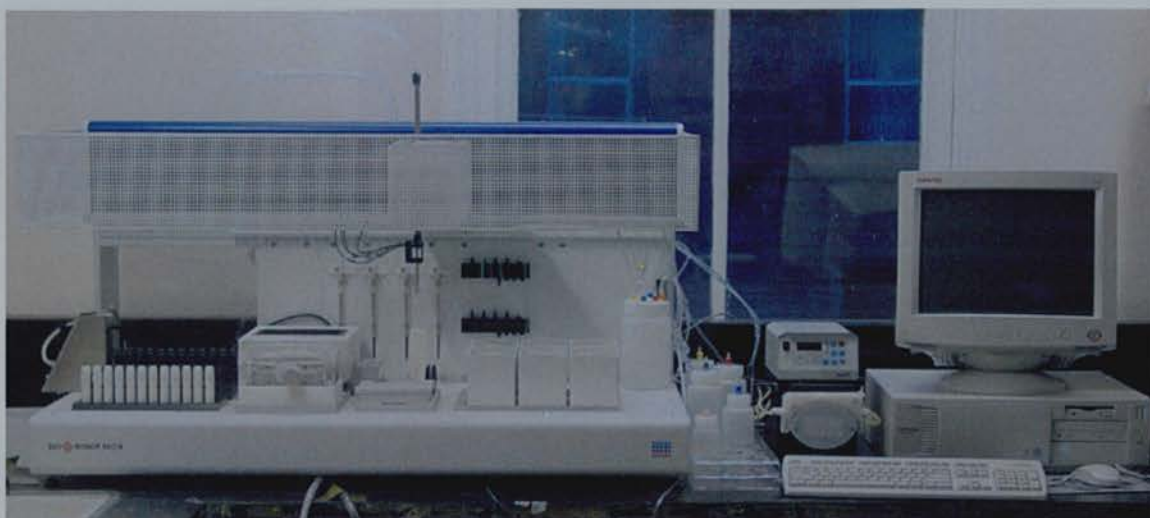


Figure 3.1 The BioRobot® 9604

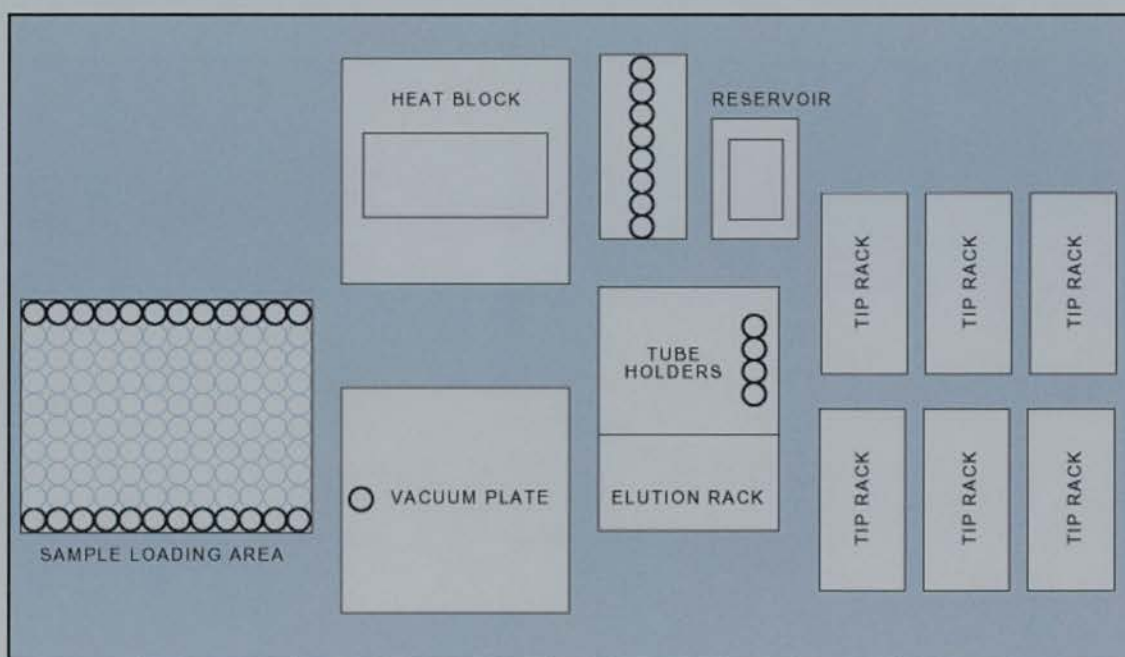


Figure 3.2 Diagrammatic representation of the BioRobot® 9604 platform

The advantages of this system include the potential for high throughput of clinical samples for DNA extraction with reliable results and consistent yields. Since a licensed protocol was not available for DNA extraction from LBC samples, computer software was modified to perform the tissue extraction protocol on the BioRobot® 9604. This work involved close collaboration with BioRobot Application Specialists and several members of the QIAGEN technical support and customer service teams. The system was extensively tested using cell pellets from approximately 4000 LBC samples. Initial processing of the LBC specimens was still done manually, as described above, and several different processing strategies were investigated.

3.3 HPV DNA detection using real-time LightCycler PCR

The commercial LightCycler™ (Idaho Technology Inc., supplied by Biogene Ltd.) combines rapid PCR technology and sophisticated computer software required for programming and product analysis (Figures 3.3). This allows DNA amplification and analysis of results simultaneously, thus producing a 'real-time' PCR system.

3.3.1 Primer design

Standard GP5+/6+ primers were synthesised by Cruachem and were HPLC-purified. Type specific primers were designed in-house by modification of the GP5+/6+ primer pair to change the primer sequences to make them specific for HPV 16 and 18. These were produced following thorough searching of the HPV Sequence Database (Los Alamos National Laboratory) in the GP5+/6+ region of the L1 gene of all sequenced HPV types and detailed analysis of product length, optimal annealing temperatures, base variations between types and the possibility of non-specific amplification. Table 3.2 shows the DNA sequences of the HPV16 and HPV18 type-specific primers.

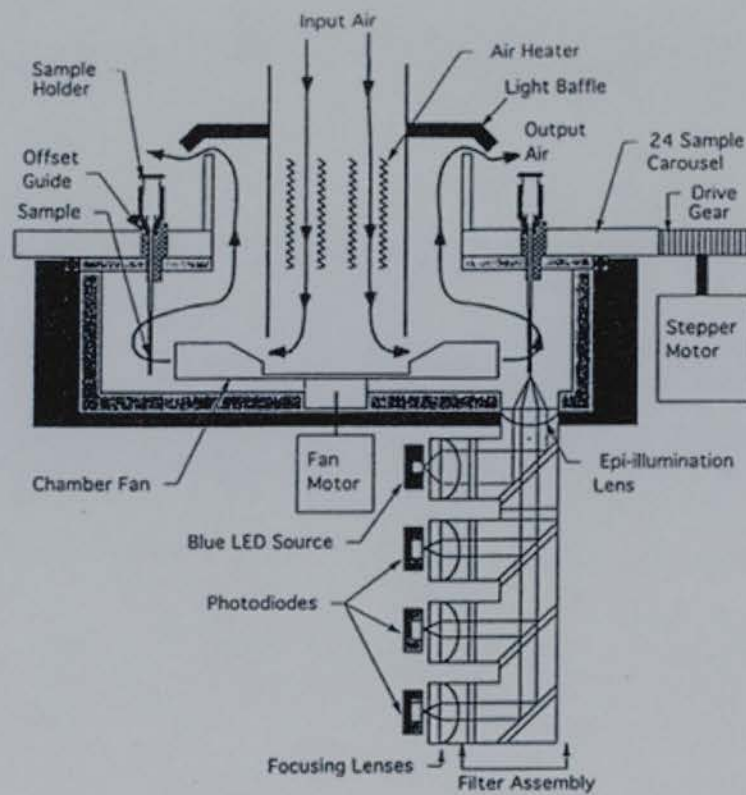


Figure 3.3 The LightCycler PCR machine linked to a personal computer and schematic of the internal operating system (Wittwer *et al.*, 1997b).

| Primer ID | Sequence |
|-----------|-------------------------------|
| TS165+ | TTGTTACTGTTGTTGATACTACA |
| TS166+ | GAAAAATAAACTGTAAATCATATTCCTCC |
| TS185+ | TTTGTACTGTGGTAGATAACCACT |
| TS186+ | GAAAAATAAACTGCAAATCATATTCC |

Table 3.2 DNA sequence of type-specific primers used for real-time LightCycler PCR

3.3.2 LightCycler PCR amplification

Since ready made LightCycler PCR master mixes containing reaction buffer, Mg^{2+} , dNTPs and Taq polymerase were available, only the fluorogenic reporter dye (SybrGreenITM), primers and template DNA needed to be added. Reaction mixes contained 0.5 μ l of 5 μ M forward and reverse primers, 0.25 μ l of 1:1000 SybrGreen I TM and 2.5 μ l of master mix containing 4mM $MgCl_2$ (Biogene Ltd.) and TaqStart antibody (Sigma–Aldrich Co. Ltd., Poole, Dorset). PCR reactions were made up in 0.5ml eppendorf tubes in the PCR ‘clean room’ and 1.25 μ l template DNA added in a separate ‘sample addition’ area. The reaction mix was then loaded into a polypropylene cup moulded to a glass capillary and sealed with a plastic stopper. After brief 10 second centrifugation step to bring the sample down to the bottom of each capillary, they were loaded into the LightCycler carousel which can hold up to 32 samples per run. The cycling profiles were optimised for each set of primers and these are detailed in Table 3.3. Positive and negative controls were included in each run. Positive control material included DNA extracted from SiHa or HeLa cells or plasmid DNA.

| PCR | Number of Cycles | Cycling conditions |
|----------|------------------|--|
| GP5+/6+ | 5 | 95°C for 0 seconds, 50°C for 3 seconds, 72°C for 3 seconds |
| | 35 | 85°C for 0 seconds, 55°C for 3 seconds, 72°C for 3 seconds |
| TS16 PCR | 5 | 95°C for 0 seconds, 59°C for 3 seconds, 72°C for 3 seconds |
| | 35 | 82°C for 0 seconds, 59°C for 3 seconds, 72°C for 3 seconds |
| TS18 PCR | 5 | 95°C for 0 seconds, 62°C for 3 seconds, 72°C for 3 seconds |
| | 35 | 85°C for 0 seconds, 62°C for 3 seconds, 72°C for 3 seconds |

Table 3.3 Optimal cycling parameters used for real-time LightCycler PCR.

3.3.3 Melting temperature analysis of PCR products

Melting temperature (T_m) analysis is performed after PCR amplification is complete. The melting temperature of DNA, defined as the temperature at which 50% of the duplicates become single-stranded, is dependant on the sequence, length and GC content. For detailed melt analysis, one capillary containing specific PCR-amplified product was loaded at a time into the LightCycler carousel. A single cycle of amplification was performed to anneal both DNA strands at 50°C for 0 seconds followed by progressively increasing the cycling temperature by thermal ramping at a transition of 0.2°C per second, to reach 85°C for 0 seconds. During this period, the fluorescent signal due to SybrGreen 1TM dissociating from the minor groove of double-stranded DNA was measured more frequently and therefore a more accurate melting temperature was assigned. For analysis of the melting curves, the computer software plots the first negative derivative of fluorescence (F) with respect to temperature (T) (dF/dT) on the y-axis against the temperature (Temp) on the x-axis. The peaks produced indicate when the maximum melting of the DNA strands occurred and thus gives the specific T_m for each DNA duplex.

The entire assay including manual DNA extraction, PCR amplification and melting temperature analysis took 3 hours.

CHAPTER 4

DETECTION OF HPV IN ARCHIVAL SMEARS USING HYBRID CAPTURE ASSAY II

4.1 Introduction

Cervical cancer is preventable providing neoplastic changes are identified prior to malignant progression and treated. It is therefore difficult to relate HPV detection to the progression of cervical disease by prospective studies since it is ethically unacceptable not to treat precancerous disease that has a high potential for progression. Retrospective follow-up studies, however, using archival Pap smears can allow the natural history of HPV infection to be studied over a considerable length of time and the relationship of HPV detection to normal and abnormal cytology to be evaluated. In most cases, HR-HPV infection has a long duration before the development of a high-grade lesion. Using archival material, Josefsson and colleagues (2000) showed that the average time between HPV detection and the development of cervical carcinoma *in situ* was 7.8 years. PCR testing of smear material has shown that most cases of newly confirmed CIN 2 or worse had previous HPV positive smears 5–6 years beforehand. More importantly, HPV DNA testing by PCR identified the development of high-grade CIN three years before the conventional diagnosis in 80% of cases and six years beforehand in two thirds of cases (Carozzi *et al.*, 2000).

Retrospective archival smear analysis relies on efficient recovery of DNA from material that has been stored for a number of years. Several workers have achieved amplification of HPV DNA from archival smears following a variety of different DNA extraction methods. As an alternative to standard phenol–chloroform extraction with ethanol precipitation of DNA, techniques such as simple freeze–thawing; proteinase K digestion with subsequent boiling (Puranen *et al.*, 1996; Jackson *et al.*, 1989); proteinase K and Tween 20 digestion followed by salting-out proteins using

NaCl (Poljak *et al.*, 1995) and guanidinium isothiocyanate (GTC)/silica have all been investigated.

The integrity of the DNA recovered from archival smears appears to be dependent on the method used and several comparative studies have attempted to test the efficacy of these methods to recover DNA from archival material. In a study by de Roda Husman *et al.*, (1995b) GTC/silica extraction was found to be most efficient at recovering DNA from archival smear material. This extraction method produced strongly positive β -globin PCR results and HPV DNA could be detected at 50–500 copies of HPV 16 per 50,000 cells. Furthermore, the GTC/silica extraction method was used by Walboomers *et al.*, (1995) to show that HPV DNA was present in 25 false negative cervical smears, from 16 women, taken up to six years before the diagnosis of cervical cancer. Such findings support the hypothesis that HPV negative cervical carcinomas do not exist (Walboomers and Meijer, 1997).

4.2 Aims of this Study

Previously, in a pilot study of 156 archival smears that had been stored for 12–13 years, it was shown that hybridisation by HCAII and amplification by GP5+/6+ PCR could be used to detect genital HPV DNA (McGoogan *et al.*, 1998). The aim of this study was to investigate the role of HR-HPV in cervical disease progression by retrospective examination of archival cervical smears over a ten-year period. The relationship between HR-HPV detection and the development of cytological and histological abnormalities was evaluated for 223 women who were regular participants in the NHS Cervical Screening Programme.

4.3 Methods

4.3.1 Selection of archived cervical smears

The University of Edinburgh Pathology Department (UEPD) laboratory computer database was searched to identify 223 women who had a cervical biopsy between 1991 and 1993, and who had at least two cervical smears in the five year periods before and after the biopsy. Most biopsies had been taken from women referred for colposcopy with a cytological abnormality (16 with low grade CIN and 87 with high-grade CIN). However, some women had been referred for colposcopy for other reasons with various findings on histology (negative $\times 10$, koilocytosis $\times 17$, polyp $\times 13$, condyloma acuminatum $\times 9$, chronic cervicitis $\times 1$, ulcer $\times 1$ and glandular dysplasia $\times 3$). In addition, histology did not always confirm the cytological findings, thus providing a group covering the whole spectrum of CIN.

Cervical smears selected from 5 years before and up to 5 years after each biopsy were removed from the UEPD archive. In total 975 Pap-stained cervical smears taken from the 223 women between 1987 and 1997 were extracted from the files. Since cervical smears are normally kept for at least 10 years, Lothian Research Ethics Committee requested that a record be made of each smear before destruction. Representative smear fields were recorded using computer-assisted PAPNET® smear screening (Section 2.2). The smears obtained were presumed to have been fixed in methylated spirits in the standard way but this was impossible to confirm.

4.3.2. Removal of cellular material from slides

After soaking the slides in xylene for 2–7 days until the coverslips became detached, cells were scraped from each slide using a sterile scalpel blade and transferred to an eppendorf tube containing 1 ml of xylene. After 45 minute incubation at room temperature, the cells were pelleted by centrifugation at 7000 rpm for 2 minutes and washed twice with 96% ethanol. Pellets were air dried at room temperature and stored overnight at -20°C . DNA extraction was performed using a modification of the GTC/silica method of Boom *et al.*, (1990) (Section 2.5.1).

4.3.3. HPV DNA detection by HCAII

Samples were tested using the HCAII high-risk probe pool containing HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68 (Section 2.6). The assay was performed according to manufacturers' instructions using a protocol adapted for 100µl volumes. The results obtained were compared to the cytology result in the same smear and with histological findings from 167 biopsy specimens.

4.3.4 HPV DNA detection by PCR

PCR amplification could not be performed from denatured HCAII specimens directly since no approved protocol was available. Attempts to amplify HPV DNA from cervical cells resuspended in Digene STM were unsuccessful since STM appeared to be inhibitory to PCR (data not shown).

Thus, to confirm the integrity of the DNA after CTC/silica extraction, PCR amplification was performed on 10µl extracted DNA using β -globin primers (Section 2.8.2). Agarose gel electrophoresis was performed to visualise the amplified PCR products (Section 2.11).

4.4 RESULTS

4.4.1. Assessment of smear cellularity

Twenty-one of 975 Pap smears were found to have unsatisfactory cytology and were not included in this analysis. To assess the amount of cellular material on each smear, the visual size of pellets scraped from the remaining 954 slides was recorded: 343 slides produced small pellets (scored +), 490 gave medium pellets (scored ++), and the remaining 121 gave large pellets (scored +++).

4.4.2. HR-HPV DNA detection using HCAII

Using HCAII, 172 HR-HPV positive results were obtained in 954 extracts from archival smears obtained from 117 patients. HR-HPV positivity increased from 10% to 30% as the size of pellet produced increased (Table 4.1).

4.4.3. HPV DNA detection by PCR

All attempts to confirm the integrity of the DNA in the archival smear extracts were unsuccessful. Following β -globin PCR amplification, no specific product was visible upon agarose gel electrophoresis.

4.4.4 HPV positivity in relation to cervical pathology

HR-HPV detection by HCAII was found to increase as the grade of cervical dysplasia increased (Table 4.2). 8.3% of smears obtained from women with no cytological abnormality were HR-HPV DNA positive. The HR-HPV detection rate increased to 41% in material extracted from smears taken from women with high-grade cytological abnormalities.

Most biopsies had been taken between 1990 and 1992, and 21% of HR-HPV positive smears were in this group (Table 4.3). From 1993 onwards, fewer HR-HPV positive results were obtained from follow-up smears due to the removal of neoplastic lesions at colposcopy with biopsy in the 1990–1992 period.

| Size of Pellet | Number Tested | HR-HPV | |
|----------------|---------------|------------|------------------|
| | | Negative | Positive |
| Small (+) | 343 | 312 | 31 (9.9%) |
| Medium (++) | 490 | 386 | 104 (21%) |
| Large (+++) | 121 | 84 | 37 (30.6%) |
| Total | 954 | 782 | 172 (18%) |

Table 4.1 Detection of HR-HPV DNA in cellular pellets scraped from fixed and stained archival smears

| Cytology Result | Total | HR-HPV | |
|--------------------------------|------------|------------|------------------|
| | | Negative | Positive |
| Within normal limits (WNL) | 560 | 515 | 45 (8.3%) |
| Borderline (B/L) | 91 | 76 | 15 (16.5%) |
| Mild dyskaryosis | 117 | 81 | 36 (30.8%) |
| Moderate or severe dyskaryosis | 186 | 110 | 76 (40.9%) |
| Total | 954 | 782 | 172 (18%) |

Table 4.2 Detection of HR-HPV in DNA extracts from 954 fixed and stained Pap smears showing different grades of dyskaryosis

| Year smear made | Total | HR-HPV Detected |
|-----------------|------------|-----------------|
| Before 1990 | 250 | 47 (18.8%) |
| 1990 to 1992 | 563 | 118 (21%) |
| 1993 to 1997 | 162 | 10 (6.2%) |
| Total | 975 | 175 |

Table 4.3 Detection of HR-HPV in DNA extracts from 975 fixed and stained archival smears made in different years

The positive and negative predictive values (PPV and NPV) of cytology and HR-HPV testing for detection of severe histological abnormalities can be calculated from the data shown in Table 4.4. The PPV of cytology alone was 70.5% (86/122) while that of HPV detection alone was only 39% (48/122). Combined cytology and HPV testing before biopsy would have increased the PPV to 78%. The NPV of a negative HR-HPV result and normal cytology was 100%.

| Cytology Result | HR-HPV | | Histology | | |
|--------------------------------|--------|-----------------|------------|----------|-----------|
| | Status | Number | CIN 2/3 | CIN1/K+ | WNL |
| Moderate or severe dyskaryosis | Pos | 40 [†] | 39* | 1 | 0 |
| | Neg | 47 | 47* | 0 | 0 |
| Mild dyskaryosis | Pos | 3 [†] | 3 | 0 | 0 |
| | Neg | 17 | 12 | 2 | 3 |
| B/L | Pos | 3 [†] | 3 | 0 | 0 |
| | Neg | 16 | 10 | 2 | 4 |
| WNL | Pos | 2 [†] | 1 | 0 | 1 |
| | Neg | 34 | 7 | 2 | 25 |
| Total | | 162 | 122 | 7 | 33 |

Table 4.4 Comparison of cervical cytology results and HR-HPV detection in relation to histological findings.

WNL = within normal limits; B/L = borderline; CIN = cervical intraepithelial neoplasia; K+ = koilocytosis

[†] values used to calculate the PPV of HPV testing alone for the detection of severe disease.
^{*} values used to calculate the PPV of cytology alone for the detection of severe disease.

To analyse these results further it is necessary to look at individual patient profiles (Figure 4.1). It can be seen that P1 and P2 developed high-grade cytological abnormalities (equivalent to HSIL) with CIN3 detected on biopsy, in the absence of HR-HPV detection. Indeed in P1, three episodes of severe abnormality were recorded during the period of study. In P3, it is also interesting to note that two HR-HPV negative smears were obtained in the two years immediately preceding HSIL or CIN3 despite earlier HR-HPV positive smears. P4 in contrast did not show any CIN on biopsy despite four previous HR-HPV positive smears. These patient profiles are representative of the 157 fully analysed cases. They span an age range of 24–31 years, reflecting a mean age of 29 in this group.

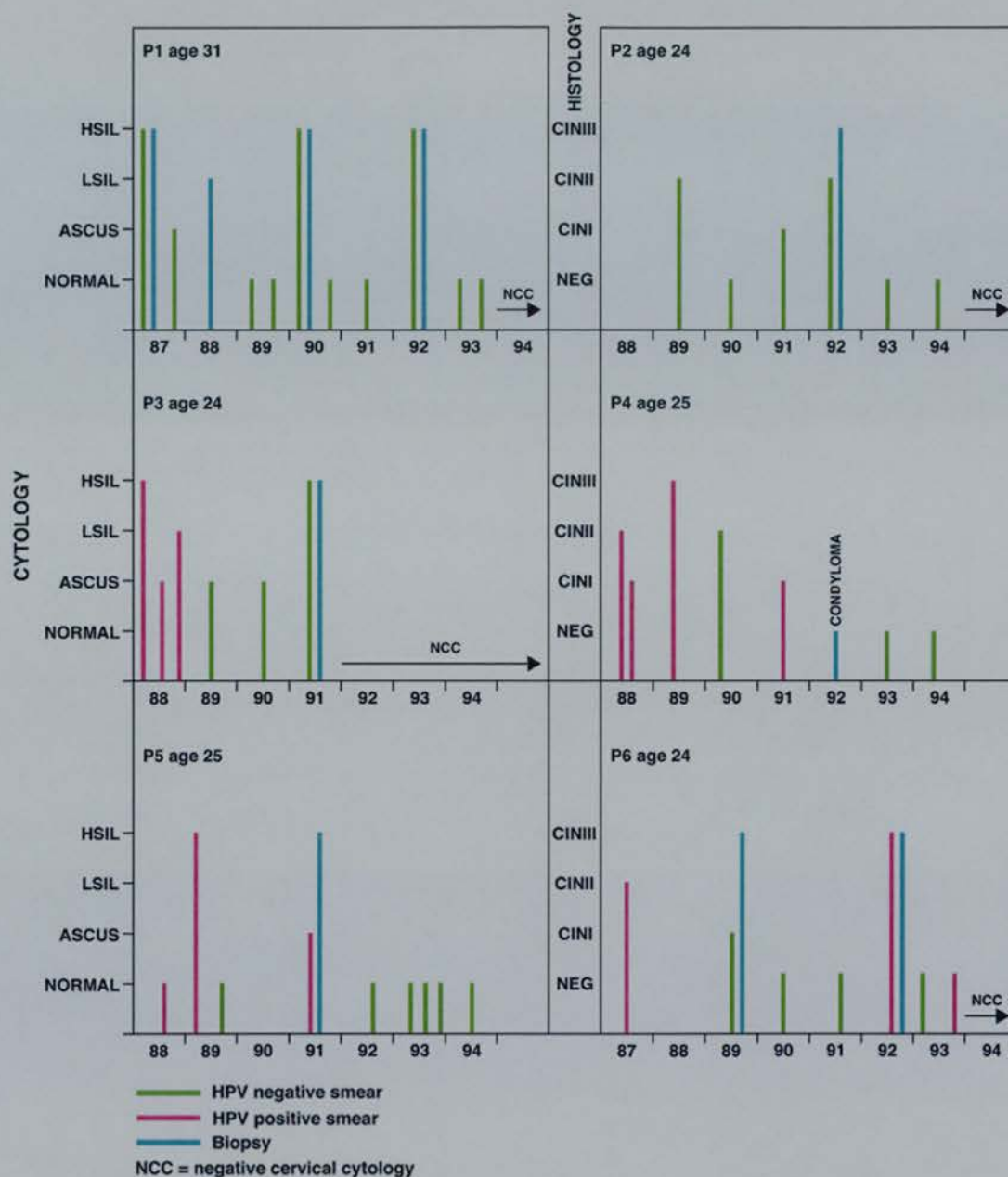


Figure 4.1 Profiles for six women showing HR-HPV DNA detection by HCAII from cellular material scraped from archival smears in relation to the cytological and histological findings

ASCUS = atypical squamous epithelial cells of undetermined significance; LSIL = low-grade squamous intraepithelial lesion; HSIL = high-grade squamous intraepithelial lesion.

4.5 Discussion

The effectiveness of cytological screening is highly dependent on the quality of taking, preparing and reading smears. Conventional cervical smears prepared on site by the smear taker are subject to great variation in technical quality and allow little control of the parameters required for optimal microscopic analysis (McGoogan and Reith, 1996). In addition, differences in sampling efficiency can produce varying amounts of cellular material on the original smear. Since the above factors could affect the quantity of DNA extracted, the visual size of the cellular pellets scraped from each slide was recorded. Interpretation of the smear itself using the naked eye was not taken into consideration since the presence of blood or mucus can influence the macroscopic appearance. Variation in the amount of material recovered from the smears did have an effect on the HPV positivity, with the highest number of HPV positive results being obtained from those smears that had greatest cellularity, as estimated by the size of the cell pellet scraped from each slide ($\chi^2_{\text{trend}} = 34.7614$ on 1 degree of freedom, $P < 0.001$ and $\chi^2 = 0.2807$ on 1 degree of freedom, $P = 0.60$).

Overall, 18% of smears were HR-HPV DNA positive. HPV positivity was found to significantly increase with increasing grade of cytological abnormality. ($\chi^2_{\text{trend}} = 115.9360$ on 1 degree of freedom, $P < 0.001$ and $\chi^2 = 0.5067$ on 2 degrees of freedom, $P = 0.78$). Rather than using HR-HPV testing as a predictor of cervical disease progression, results suggested that HPV testing would have a greater role in *excluding* severe disease, since the negative predictive value of a negative HR-HPV test and normal cytology was 100%. Bollen and colleagues (1997) obtained similar results, with negative HPV results selecting for absence of high-grade intraepithelial lesions. However, another study suggested that 100% of cone biopsies showing CIN 2–3 would be identified if they were HPV positive but showing low-grade abnormalities (Fait *et al.*, 1998). In the Edinburgh archival smear study this would not have been the case.

Colposcopic intervention was largely successful in removing much HPV infection, shown by the significant decrease in the HPV positivity rate in smears after 1993, when treatment at colposcopy would have been given ($\chi^2 = 14.2722$ on 1 degree of

freedom, $P < 0.001$). This has similarly been shown in a study by Bollen *et al.*, (1997). None of the 10 women who had subsequent HPV positive test results during 1993 to 1997 had further cytological abnormalities. However, it appears that half of these women had very low-level HPV infection as shown by RLU indices of between 1.41 and 1.7. Indeed, these may signify false positive HCAII results.

In this study, HPV positivity was considerably higher than that obtained in our pilot study using 13 year old smears and HCAI, in which only 2.3% with normal cytology, 4.5% with low-grade abnormalities and 21.7% of smears with high-grade cervical disease were HR-HPV positive (McGoogan *et al.*, 1998). The higher percentage of positives obtained in this group reflects the greater sensitivity of HCAII and the length of storage time for the smears (maximum storage 10 years). However, the HPV positivity rate for smears showing high-grade lesions was lower than obtained in later studies with liquid-based cytology samples (see Chapters 6 and 7). Possible reasons for this reduction in the sensitivity of HPV testing include low copy number of HPVs rendering the extracted DNA undetectable by HCAII. The possibility that some samples that were HR-HPV DNA negative may contain low-risk HPV genotypes also cannot be excluded but HCAII testing using the low-risk probe cocktail was not performed as part of this study.

PCR amplification of DNA from archival smears was problematic. Preliminary attempts to achieve a reproducible PCR assay were unsuccessful, which was attributed to the presence of inhibiting substances in the extracts that prevented nucleic acid amplification occurring. It has been documented that the haematoxylin solution and aluminium sulphate components of the Papanicolaou stain can interfere with PCR (Chen *et al.*, 1996). Even positive control material spiked with DNA extracted from archival smear material resulted in no subsequent amplification of the target DNA, although this could be partly overcome by dilution of the DNA before addition to the PCR reaction. In other studies, dilution of the digested cellular DNA (1:100) did not appear to remove the inhibitors but PCR analysis was possible if the Pap smears were firstly destained with 1% HCl or if phenol-chloroform extraction was performed (Chen *et al.*, 1996). Phenol-chloroform extraction appeared to be

effective at removing inhibitors even if all the stain was not removed (Gall *et al.*, 1993). In HCAII testing, inhibition was not apparent but it is conceivable that the lower HPV positivity obtained may also be due to the inhibitory effects of residual stain. Although, in a study by McDonald *et al.*, (1999) electrophoresis of DNA from stained smears was degraded to a much greater extent than DNA extracted from unstained smears.

Further PCR protocol development was not pursued as part of the study reported here and a protocol was not developed to allow PCR testing on denatured HCAII samples directly. If this had been possible, more HPV positive cases might have been detected using PCR amplification, which is more sensitive than HCAII (Cavuslu *et al.*, 1996). In a previous pilot study involving 156 archival smears that had been stored for 12–13 years, we found that both HCAII and GP5+/6+ PCR could be used for the detection of HPV DNA (McGoogan *et al.*, 1998). Nevertheless, in a small study of 11 archival smears by Puranen *et al.*, (1996) four out of five smears showing high-grade abnormalities remained HPV DNA negative by PCR. Similarly Carozzi *et al.*, (2000) found that 21 of 92 (22.8%) smears showing high-grade disease did not appear to be preceded by HPV infection. This was confirmed in the study reported here, but this is unlikely to be attributed to the age of the smears since other patients did have HR-HPV positive smears from similar dates. The lack of detectable HPV could be attributed to either the small amount of DNA present (<1 copy/cell) in these smears or deletion of the L1 region due to viral integration. DNA in archival material stored for long periods may also be subject to degradation, which could cause generation of false negative HPV results. Additionally, the quality of fixation of the original smear could affect cell preservation over extended time periods. In a study by de Roda Husman *et al.*, (1995b), storage time was found to have an effect on the size of PCR target that could be amplified, since PCR targets of more than 200bp could not be amplified from smears that had been stored for a long time. Similarly, Carozzi *et al.*, (2000) found the proportion of HPV DNA positive smears among cases only decreased slightly with increased storage time (less than or more than 4 years).

Another critical factor for the detection of HPV DNA in archival smears appears to be the method used for DNA extraction. The GTC/silica extraction method is very laborious and involves many manipulation steps during which the DNA could be lost. A recent comparative study involving two proteinase K-based treatments and two GTC-based assays found a GTC/silica-based column method, named the High Pure PCR Template Preparation (HPPTP) assay, to be the most effective at DNA extraction from archival cervical smears resulting in a larger amplifiable DNA yield. These commercially available extraction columns, gave 15–44% extraction efficiency whereas the GTC/silica method of Boom *et al.*, (1990) gave an efficiency range of only 1–3% in comparison (Jacobs *et al.*, 2000).

Due to some of the problems outlined above, the use of archival smear material is likely to underestimate HPV positivity compared with that obtained by using fresh material. However increased HPV DNA positivity in archival smears has been achieved by other workers using two sets of consensus PCR primers for HPV detection. Following DNA extraction using a QIAGEN tissue kit protocol, Carozzi and colleagues (2000) used consensus primers pU-1M and pU-2R (Fujinaga *et al.*, 1991) to amplify a 233–268bp fragment within the E6–E7 region of HPV 16, 18, 31, 33, 52 and 58. McDonald and colleagues (1999) obtained strong PCR positive results using the consensus E1 primers CPI-IIG (Tieben *et al.*, 1993) in some sample dilutions that produced PCR negative results with the HPV L1 primers GP5/6. CPI-IIG, that amplify a 188bp fragment of the E1 ORF of a broad spectrum of HPV types, have also been used successfully in another study resulting in HPV DNA detection in all 13 abnormal smears that had been stored for 4 years (Svare *et al.*, 1998). A possible explanation for the above findings may be improved DNA detection due to more efficient amplification of a smaller HPV DNA target. Jacobs *et al.*, (2000) found that β -globin PCR positivity decreased with the increasing length of amplified PCR product. Now, primer systems are available for PCR amplification of smaller DNA fragments. For example, the SPF10 primer set will amplify a 65bp fragment of the HPV L1 open reading frame (Kleter *et al.*, 1998). These primers may be more efficient at amplification from archival DNA, which has been degraded over

time. However, due to lack of time and resources, this could not be investigated in this study.

HPV positivity might also have been increased if re-extraction of samples could have been attempted, since de Roda Husman *et al.*, (1995b) found that performing an additional lysis and elution step on β -globin PCR negative samples increased the HPV positivity rate from 65% to 96%. A further round of extraction was found to increase the efficiency of HPV DNA extraction even further, from 96% to 98%. In addition, detection of HPV directly in paraffin-embedded biopsy tissue from the women in the study reported here may have provided information regarding the natural history of cervical HPV infection.

4.6. Conclusion

HPV testing by HCAII is a suitable screening tool for archival smears. Extraction of DNA from archival smears was shown to be problematic for PCR amplification, confirming several other studies in recent years. With the advent of new extraction methods which remove inhibitors and PCR primers that amplify shorter target sequences, there remains a role for longitudinal studies of archival material to provide useful information on the natural history of HPV infection and the relevance of its detection.

Although the PPV of a positive HR-HPV result for a CIN 2–3 biopsy was lower than cytology (39% vs 70.5%), the combined results would have been of value in ensuring the appropriate women were referred for colposcopy. More importantly, the NPV of a normal biopsy following a negative HR-HPV test in the presence of cervical cytology within normal limits was 100%. Thus, the lack of HPV detection may be a better predictor than cytology for the absence of subclinical HPV-associated cervical neoplasia although both HPV negativity and normal cytology remain necessary to give a 100% negative predictive value. Such HPV negative women with normal cytology are at low-risk of developing a high-grade cervical lesion over the next 3 to 4 years (Rozendaal *et al.*, 1996).

CHAPTER 5

HPV DETECTION AND IDENTIFICATION OF HPV GENOTYPES USING DNA SEQUENCE ANALYSIS IN CERVICAL SCRAPE SAMPLES FROM HIV-SEROPOSITIVE WOMEN

5.1 Introduction

Before the onset of the HIV epidemic in the 1980s, it had already been established that immunosuppressed individuals were particularly susceptible to HPV infection. For example, cell mediated immunosuppression due to organ transplantation, chemotherapy and/or radiation therapy increased the risk of developing HPV infections, particularly genital warts (Benton and Arends, 1996). In addition, transplant patients receiving immunosuppressive drugs had an increased risk of CIN (Ozsaran *et al.*, 1999). HIV is now a major cause of cell-mediated immunodeficiency worldwide due to the cytopathic effects of HIV on T-lymphocyte 'helper' (CD4⁺) cells of the immune system. This makes the host susceptible to a variety of opportunistic infections since effective immune responses are suppressed.

In comparison to the general female population, HIV-seropositive women have been shown to have an increased risk of developing invasive cervical carcinoma, especially HIV-infected intravenous drug users (Serraino *et al.*, 1999) thus supporting the inclusion of invasive cervical carcinoma as an AIDS-defining condition (Centres for Disease Control, 1992). Several studies have consistently shown that HIV-seropositive women, compared to HIV-seronegative women, show increased HPV prevalence and persistence with increased development of CIN (Table 5.1), which is more aggressive and progresses more rapidly to invasive cancer. However, the exact prevalence of CIN in HIV-seropositive women is difficult to determine due to the different populations studied and variations in the methodology used. Comparisons between different studies can be very difficult since cervical abnormalities may have been assessed by cytology, histology or HPV status.

| Study | Location | Study Group | Cervical Assessment | Cytological Abnormality | HPV Testing | HPV Prevalence |
|---------------------------------|-------------|-----------------------|----------------------|-------------------------|-------------|----------------|
| Ahdieh <i>et al.</i> , 2000 | USA (ALIVE) | 184 HIV+ 84 HIV– | Cytology | 13.4% 2.4% | PCR | 69.6% 26.2% |
| Ellerbrock <i>et al.</i> , 2000 | USA | 328 HIV+ 325 HIV– | Histology | 20% 5% | PCR | 61% 23% |
| Moscicki <i>et al.</i> , 2000 | USA | 133 HIV+ 55 HIV– | Cytology | 36% 12% | PCR | 77.4% 54.5% |
| Massad <i>et al.</i> , 1999 | USA (WIHS) | 1713 HIV+ 482 HIV– | Cytology + histology | 38.3% 16.2% | PCR | 63.2% 30.4% |
| Maiman <i>et al.</i> , 1998 | USA | 248 HIV+ 220 HIV– | Cytology + histology | 32.9% 7.6% | PCR | 75.1% 46.5% |
| Rezza <i>et al.</i> , 1997 | Italy | 135 HIV+ 101 HIV– | Cytology | 35.6% 8.9% | PCR | 40% 32% |
| Shah <i>et al.</i> , 1997 | USA | 150 HIV+ 72 HIV– | Cytology | 18.4% 1.4% | PCR | 69.3% 26.4% |
| Capiello <i>et al.</i> , 1997 | Italy | 135 HIV+ 101 HIV– | Cytology | 35.8% 9.2% | PCR+RFLP | 40.3% 29.6% |
| Miotti <i>et al.</i> , 1996 | Africa | 129 HIV+ 155 HIV– | Cytology | 15% 7% | PCR | 25.5% 23% |
| Langley <i>et al.</i> , 1996 | USA | 140 HIV+ 619 HIV– | Cytology | 9.8% 6.8% | PCR | 56% 40% |
| Schrager <i>et al.</i> , 1989 | USA | 35 HIV+ 23 HIV– | Cytology | 31% 4% | Cytology | 26% 4% |

Table 5.1 Summary of studies investigating cervical cytology and HPV prevalence in HIV–seropositive and HIV–seronegative women

Some studies also lack comparison between HIV-infected women and a parallel control population of HIV non-infected women with similar behavioural characteristics. Moreover, there is an ethical requirement for treatment if significant cervical disease is identified. However, even after treatment by loop electrosurgical excision or ablation, cervical lesions in HIV-positive women are more likely to recur or persist (Wright *et al.*, 1994b). Within two years of treatment, 34% may have recurrent cervical lesions (Beattie *et al.*, 1994) but as many as 82% HIV-positive immunosuppressed women have been shown to have recurrent lesions after treatment for CIN by conization (Six *et al.*, 1998). Recurrence of CIN in HIV seropositive women after standard ablative treatment has been shown to be related to increasing immunosuppression (Maiman *et al.*, 1993), with recurrence rates at 3 years reported to be above 85% for women with CD4 counts less than 200 cells/ μ l (Fruchter *et al.*, 1996).

Despite this, an increased cervical HPV detection rate has not always been found when HIV immunosuppression increases and some studies have found no apparent increase in cervical neoplasia in HIV-infected women if adequate screening and treatment programmes have been in place (Fruchter *et al.*, 1996). Such findings have prompted some authors to suggest that the role of HIV as an independent risk factor for CIN is not yet proven (Norman and McCarthy, 1994). Although both HIV and HPV are sexually transmitted, it appears that the association between them is complex and cannot be fully explained by the level of immunosuppression alone.

Various studies have attempted to clarify the demographic or behavioural risk factors associated with increased prevalence of HPV in HIV-infected women, with mixed outcomes. A large national multicentre study of 1713 HIV-infected women (the Womens Interagency HIV Study (WIHS)) found that factors linked to sexual and reproductive history (e.g. number of sexual partners within six months of enrollment), HPV infection (e.g. abnormal cytology history) and HIV infection (e.g. CD4 count and HIV RNA level) all influenced the risk for abnormal cytology (Massad *et al.*, 1999). Smaller studies have also suggested that sexual behavioural characteristics are associated with increased HPV detection in HIV-seropositive

women, including the number of sex partners and number of pregnancies (Piper *et al.*, 1999; Temmerman *et al.*, 1999). Smoking (more than 20 cigarettes per day) has also been shown to be a significant risk factor for the development of cervical disease in HIV-infected women also (Heard *et al.*, 1997). In contrast, Klein *et al.*, (1994) failed to show any association between several demographic or behavioural variables and the risk of cervical abnormalities in HIV-seropositive women. Nonetheless, it should be remembered that in addition to such classical risk factors for the progression of cervical disease in HIV positive individuals, other variables such as the particular HPV genotype(s) involved and the duration of HPV infection may also be important.

The mechanism through which HIV-associated immunosuppression increases the risk for CIN is not well understood. Some workers have suggested that HPV and HIV interact at a molecular level where the tat-1 gene (a regulatory protein of HIV-1) enhances HPV gene expression (Chopra and Tyring, 1997). It has also been suggested that the development of many HIV-associated neoplasias is influenced by microsatellite alterations in chromosome 3p, which reflects widespread genomic instability. Microsatellite alterations have been found at much greater frequency in CIN in HIV-infected women although the underlying mechanism is unknown (Wistuba *et al.*, 1999). Possible effects of immunosuppression on the natural history of HPV include the reactivation of latent HPV infections and the establishment of chronic or persistent infections. A very strong link between HPV detection and CD4⁺ lymphocyte count has been shown since HPV appears to be detected more frequently and more persistently in HIV-infected women with reduced CD4 counts (Vernon *et al.*, 1995; Sun *et al.*, 1997; Palefsky *et al.*, 1999) with 90% of cases with CD4 counts below 200 cells/ μ l harbouring HPV DNA (Johnson *et al.*, 1992). The efficiency of HIV suppression may even influence the infecting HPV type since in a study by Johnson and colleagues (1992), half of the HIV infected women with CD4 counts <200 cells/ μ l were found to harbour HPV 18, compared to only one of 22 women with CD4 counts above 200 cells/ μ l. However, this finding has not been confirmed by others (Branca *et al.*, 2000). In addition, HIV-infected women with CD4 counts of 200–500 cells/ μ l have a 3-fold increased risk of developing cytological

abnormalities. When immunosuppression increases further and CD4 counts fall to below 200 cells/ μ l these women have 4-fold increased risk of abnormal cytology (Six *et al.*, 1998). There is also a 4-fold increase in the number of women with untreated low-grade cervical disease that will not regress to normal (Delmas *et al.*, 2000).

To improve the immune status and prognosis of HIV-infected individuals, potent antiretroviral drugs have been developed. Nowadays, these are generally administered in combinations of three or more agents and usually consist of one protease inhibitor combined with at least two reverse transcriptase inhibitors. Such combinations are known as highly active antiretroviral therapy (HAART). There are currently eighteen approved drugs for HIV infection (Internet citation: www.aidsmeds.com/lessons/Drug_Chart.htm). Some studies have suggested that there is a protective effect provided by antiretroviral therapy against the occurrence of cervical disease in HIV seropositive women. Effective HAART regimes have the potential to reduce the plasma HIV RNA viral load, which would allow some restoration of immune function. This could conceivably also reduce HPV persistence and the degree of CIN found in HIV-infected women. Alternatively if HAART has little or no impact on the regression of cervical disease then the prevalence of cervical neoplasia is likely to increase as patient survival time increases.

5.2 Aim of this study

The main objective of this work was to monitor the presence of HPV infection in relation to cervical pathology in HIV-seropositive women in Edinburgh. Over a four-year period, HPV testing was performed on collected cervical scrape samples using HCAII and GP5+/6+ PCR. Direct DNA sequencing of the GP5+/6+ PCR product was investigated as an HPV genotyping tool. Data on cervical cytology, histology, HIV viral load results, antiretroviral drugs and CD4 cell counts was also collected to allow the natural history of HPV in this population to be studied.

5.3 Methods

5.3.1 Study population

Women were enrolled from the cohort of 164 HIV infected women who attend the colposcopy clinic at the Edinburgh Regional Infectious Diseases Unit. This cohort of HIV-infected women was one of the first well-documented groups where infection arose largely as a result of intravenous drug use (IVDU) and needle sharing. The majority seroconverted over a fairly short period of time in the mid-1980s. The women are all of Caucasian origin and are representative of the homogeneous population resident in Lothian.

The stable well-defined nature of this group and the regular attendance of most at clinics make it an ideal cohort for a longitudinal study of natural history of HIV-associated HPV infection. Thus, a prospective study to monitor the presence and persistence of HR-HPV types in these HIV-infected women has been ongoing since 1996. A clear association between abnormal cervical cytology and HIV infection has previously been reported with some degree of CIN found in 35% of these HIV-seropositive women (Johnstone *et al.*, 1994; Beattie *et al.*, 1994).

5.3.2 Specimen collection

At the same time as cervical sampling for conventional cytological analysis, cervical brush specimens were collected in either 10mls PBS solution (n=198) or CytoRich preservative fluid (AutoCyte Inc, Burlington, NC) (n=185). All specimens were collected by Dr. Gerry Beattie, Consultant Gynaecologist.

383 cervical brush specimens were collected from 114 women (age range 20–55 years; mean age 33.6yrs) who attended regularly at approximately six-monthly intervals. Specimen collection occurred between 1996 and 2000. A single cervical sample was obtained from 34 women. 62 women had between two and six specimens collected and seven or more samples were obtained from 18 women.

5.3.3 Specimen processing

Specimen processing was performed by dividing each sample into two equal volumes. After centrifugation at 3660 rpm for 15 minutes, one aliquot was resuspended in 150µl of Digene STM/DNR mix for HPV testing by HCAII, and the other was resuspended in 180µl of ATL Buffer (QIAGEN Ltd., West Sussex. UK) for DNA extraction. All samples were then stored at –20°C until HPV testing was performed.

5.3.4 Detection of HPV DNA and identification of HPV genotypes

HCAII testing was performed according to manufacturers' instructions using the high-risk probe pool for detection of HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68 (Section 2.6). Only if the DNA sequencing result identified the presence of HPV 6, 11, 42, 43 or 44 was the low-risk HCAII probe pool used to confirm the result.

DNA extraction was performed using the QIAamp® DNA Mini Kit Tissue protocol (Section 2.5.2). Consensus GP5+/6+ PCR amplification was performed as described in Section 2.8.1 and PCR products were analysed in 2% agarose stained with ethidium bromide. If non-specific product amplification was observed, the specific product band was excised from the gel and purified using QIAquick® gel extraction procedure (Section 2.12). After repeat GP5+/6+ PCR amplification and gel electrophoresis, a single specific product was then observed which was used for direct sequencing.

Both strands of selected PCR products were sequenced by DNASHEF Technologies (Department of Haematology, Royal Infirmary of Edinburgh) using ABI Prism® BigDye™ Terminator Cycle sequencing with Ready Reaction Kits (Section 2.14). GP5+/6+ primers were used as the sequencing primers and the sequences from both strands were aligned and compared to those of known HPV types as described in Section 2.14. If the sequence could not be interpreted due to multiple peaks present at the same positions, then this was considered to signify a mixed infection with more than one HPV genotype represented by two or more DNA fragments in the

PCR product solution. The RLBA was used to show the exact HPV genotypes present when multiple HPV infection was suspected (Section 2.10).

5.3.5 HIV-1 viral load testing and CD4 cell counts

Plasma HIV RNA level is used as a predictor of clinical outcome for HIV-infected individuals and CD4 lymphocyte counts are used as a marker of immunosuppression. In general, HIV-infected persons with CD4 counts of <200 cells/ μ l are considered functionally immunosuppressed whereas individuals with CD4 counts above 200 cells/ μ l are relatively immunocompetent (Johnson *et al.*, 1992).

HIV-1 RNA was measured diagnostically at the RCVL by the HIV-1 Amplicor assay (Roche Diagnostics Ltd., Lewes, East Sussex) and the results made available. HIV viral load testing began at RCVL in June 1996 using an assay with a lower limit of detection of 400 copies/ml of plasma. From June 1999, an ultra-sensitive assay was used when clinically requested which had a lower limit of detection of 50 copies/ml plasma. CD4 counts were made available from patient results held at RCVL.

5.3.6 Cytology and histology results

Cervical smears were assessed under standard diagnostic conditions (Section 2.3). Details of cytological assessment and histological evaluation were made available from records held at the UEPD. Patients with histological evidence of CIN of any grade were treated using either cold coagulation or, more recently, loop diathermy in the conventional manner.

Ethical permission for this study was granted by Lothian Health (LREC/1999/4/78). The DNA sequencing work was supported by Royal Infirmary of Edinburgh Small Project Award 98/13. The RLBA was kindly provided by Roche Molecular Systems Inc. (Alameda, CA).

5.4 Results

5.4.1 Correlation between cytology and HPV DNA detection

A total of 383 specimens were collected during the duration of this study. Thirty two specimens were omitted from further analysis as there was either no corresponding Pap smear available (n=30) or no cytology result available (n=2). A further five samples were lost and could not be tested using HR-HCAII so these were also excluded.

225 of the 335 samples (65%) collected from HIV-infected women with satisfactory Pap smears had no evidence of cervical abnormalities and 110 (32.8%) had abnormal smear results. 46 of these (13.7%) were associated with a B/L smear result; 37 (10.9%) with mild dyskaryosis; 20 (5.8%) with moderate dyskaryosis and 7 (2.1%) with severe dyskaryosis.

HR-HPV DNA was detected by HCAII in 155 of 346 specimens (44.8%). HR-HPV positivity rose from 25.7% of samples associated with normal cytology to 95% of samples associated with moderate dyskaryotic smears and 100% of samples associated with severe dyskaryotic smears. The degree of abnormality observed cytologically together with the percentage of HR-HPV positive samples is shown in Table 5.2.

More than one cervical sample was obtained from 80 women. The HPV positivity overall was similar whether first, last or all samples were considered. No high-grade dyskaryosis was observed in the last specimens obtained.

| Cytology | FIRST SPECIMENS | | | LAST SPECIMENS | | | ALL SPECIMENS | | |
|--------------|-----------------|-------------------|-----------|----------------|-------------------|-----------|---------------|--------------------|------------|
| | HR-HPV | | | HR-HPV | | | HR-HPV | | |
| | n | POS (%) | NEG | n | POS (%) | NEG | n | POS (%) | NEG |
| U/S | 6 | 2 (33.3) | 4 | 1 | 1 | 0 | 11 | 3 (27.3) | 8 |
| WNL | 66 | 18 (28.1) | 46 (2NT) | 53 | 11(21.1) | 41 (1NT) | 225 | 58 (25.3) | 167 (4NT) |
| B/L | 19 | 13 (72.2) | 5 (1NT) | 10 | 8 (80.0) | 2 | 46 | 35 (74.5) | 11 (1NT) |
| Mild | 9 | 9 (100) | 0 | 12 | 9 (75.0) | 3 | 37 | 33 (89.2) | 4 |
| Mod | 5 | 4 (80.0) | 1 | 0 | 0 | 0 | 20 | 19 (95.0) | 1 |
| Sev | 3 | 3 (100) | 0 | 0 | 0 | 0 | 7 | 7 (100) | 0 |
| TOTAL | 108 | 49 (46.7%) | 56 | 76 | 29 (38.7%) | 46 | 346 | 155 (44.8%) | 191 |

Table 5.2 HR–HPV DNA detection in cervical brushing samples from HIV –infected women

U/S = unsatisfactory; WNL = within normal limits; B/L = borderline; Mild = mild dyskaryosis; Mod= severe dyskaryosis; Sev = severe dyskaryosis; n = number of specimens; NT= not tested

5.4.2 Comparison of GP5+/6+ PCR and HCAII for detection of HPV DNA

One hundred and thirty-two specimens (from 66 women) were tested by both HR–HCAII and GP5+/6+ PCR methods. A single specimen had been collected from 22 women, two specimens from 24 women and three or more from 20 women. Sixty of 132 (47%) specimens were found to be HPV positive by HR–HCAII and 78 specimens (59%) were found to be HPV positive by consensus GP5+/6+ PCR (Table 5.3). On direct comparison of HR–HCAII and GP5+/6+ PCR data, concordant results were seen in 96 (72.7%).

| | | GP5+/6+ PCR | | TOTAL |
|-----------|-----|-------------|-----------|------------|
| | | POS | NEG | |
| HR–HCA II | POS | 51 | 9 | 60 |
| | NEG | 27 | 45 | 72 |
| TOTAL | | 78 | 54 | 132 |

Table 5.3 Comparison of HCA II (high–risk probe pool only) and GP5+/6+ PCR for HPV detection

5.4.3 Direct DNA Sequencing of GP5+/6+ PCR products

Of the 78 GP5+/6+ PCR positive specimens from 44 women, 73 were subject to DNA sequence analysis (using the GP5+/6+ primers as the sequencing primers) to identify the exact HPV type present (Table 5.4). Four PCR positive samples were not sequenced and one sample failed to generate interpretable sequence data (sequence failure) but was later tested by RLBA. Abnormal cytology was associated with about one third (21/62) of the specimens in this group (B/L 6.5%; mild dyskaryosis 14.5%; moderate dyskaryosis 9.7%; severe dyskaryosis 3.2%).

Many HPV genotypes previously isolated from the genital mucosa were found. The majority of HPV DNA positive samples contained a single genotype (79%) (Figure 5.1). Twenty-nine HR-HPVs, 14 LR-HPVs and 15 novel HPV genotypes were identified. However, in 15/73 samples, multiple HPV infection was indicated. HPV 16 and HPV 18 were the most common HR-HPV genotypes but the HPV 16 positivity rate was only 9.5%. The most prevalent LR-HPV genotypes were HPV 42 and JC9710. In total, 22 different HPV genotypes were identified in single infections and analysis of these genotypes identified in normal and dysplastic smears is shown in Table 5.4. Both cases of severe dyskaryosis were found to harbour HPV 18 DNA. Sequence analysis of 25 of 27 HR-HCAII negative, GP5+/6+ PCR positive samples showed that 18 samples contained low-risk or novel HPV genotypes that would not be detected by the HCAII high-risk probe cocktail (HPV 42 four times, JC9710 four times, HPV 11 twice, HPV 32 twice, HPV 6b, HPV 54, , HPV 73, CP8304 (Figure 5.2), CP1608 and HPV 67). Nine of these 18 samples were then tested using the HCAII low-risk probe cocktail. All four HPV 42 isolates and the HPV 6b isolate gave LR-HCAII positive results. Samples containing HPV 32, HPV 73 and JC9710 gave LR-HCAII negative results since these types are not included in the probe cocktail.

Sequence analysis of 15 samples suggested that one or more HPV genotype was indicated, as sequence data showed multiple nucleotide peaks at the same positions (Figure 5.3). However, the exact HPV genotypes present could not be confirmed by this method. This group contained six samples that were HR-HCAII negative but GP5+/6+ PCR positive. Three of these were tested using the HCAII low-risk probe cocktail: two were LR-HCAII negative and one sample gave a LR-HCAII positive result. RLBA testing was performed on all six samples containing more than one HPV genotype for clarification.

| | CYTOLOGY | | | | | | TOTAL |
|------------------------|-----------|----------|-----------|----------|----------|-----------|------------|
| | Unknown | U/S | WNL | B/L | Mild | Mod & Sev | |
| PCR | | | | | | | |
| HPV neg | 7 | 1 | 41 | 0 | 0 | 0 | 49 |
| HPV pos | 11 | 2 | 44 | 4 | 9 | 8 | 78 |
| TOTAL | 18 | 3 | 85 | 4 | 9 | 8 | 127 |
| DNA SEQUENCING | | | | | | | |
| Not sequenced | 0 | 0 | 5 | 0 | 0 | 0 | 5 |
| Single infection | 11 | 1 | 30 | 4 | 6 | 6 | 58 |
| Multiple infection | 0 | 1 | 9 | 0 | 3 | 2 | 15 |
| TOTAL | 11 | 2 | 44 | 4 | 9 | 8 | 78 |
| HIGH-RISK HPV | | | | | | | |
| HPV 16 | 3 | | 1 | | 2 | 1 | 7 |
| HPV 18 | 4 | | 1 | | | 2 | 7 |
| HPV 39 | 1 | | | | 1 | | 2 |
| HPV 45 | | | 1 | | 1 | | 2 |
| HPV 52 | | | | | | 1 | 1 |
| HPV 56 | 1 | | | 1 | | | 2 |
| HPV 58 | | | 2 | 1 | | | 3 |
| HPV 31 | | | 1 | | | | 1 |
| HPV 35 | 1 | | | | | | 1 |
| HPV 51 | | | 1 | | | 1 | 2 |
| HPV 54 | | | 1 | | | | 1 |
| TOTAL | 10 | 0 | 8 | 2 | 4 | 5 | 29 |
| RARE/NOVEL HPVs | | | | | | | |
| HPV 70 ¹ | | | | 1 | | | 1 |
| HPV 73 ² | | | 1 | | | | 1 |
| CP6108 | | | 2 | | | | 2 |
| CP8304 | 1 | | 4 | | 1 | 1 | 7 |
| MM7 ³ | | 1 | 2 | | | | 3 |
| HPV 67 | | | | | 1 | | 1 |
| TOTAL | 1 | 1 | 9 | 1 | 2 | 1 | 15 |
| LOW RISK HPVs | | | | | | | |
| HPV 6b | | | 1 | | | | 1 |
| HPV 11 | | | 2 | | | | 2 |
| HPV 32 | | | 3 | | | | 3 |
| HPV 42 | | | 3 | 1 | | | 4 |
| JC9710 | | | 4 | | | | 4 |
| TOTAL | 0 | 0 | 13 | 1 | 0 | 0 | 14 |

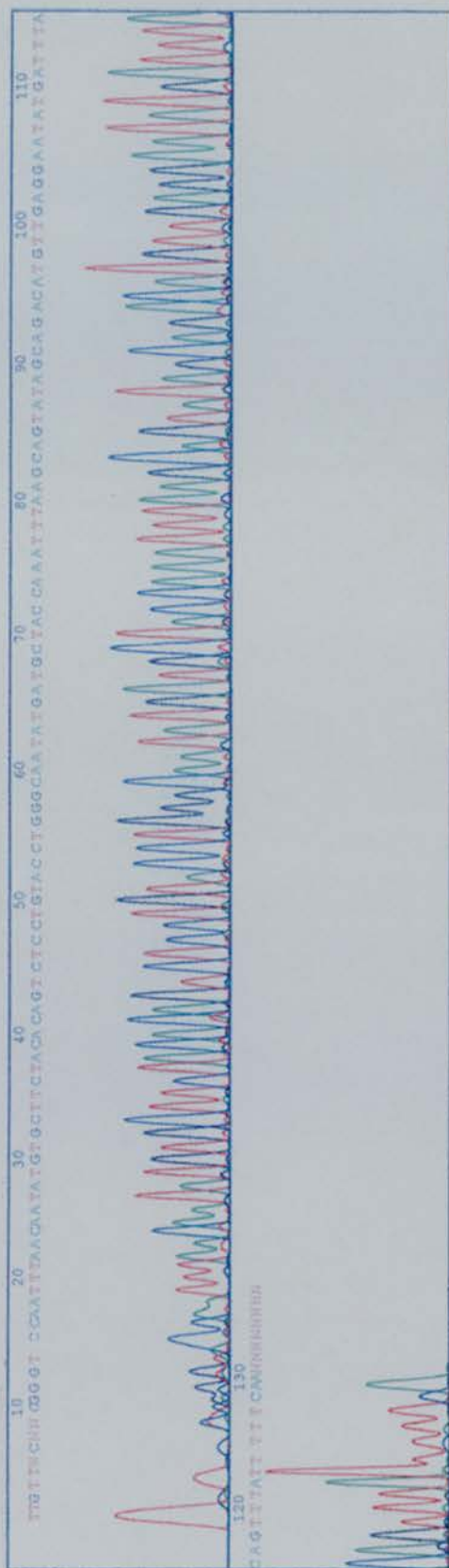
Table 5.4 DNA sequencing results in relation to cytology.

U/S = unsatisfactory; WNL = within normal limits; B/L = borderline; Mild = mild dyskaryosis; Mod = moderate dyskaryosis; Sev = severe dyskaryosis

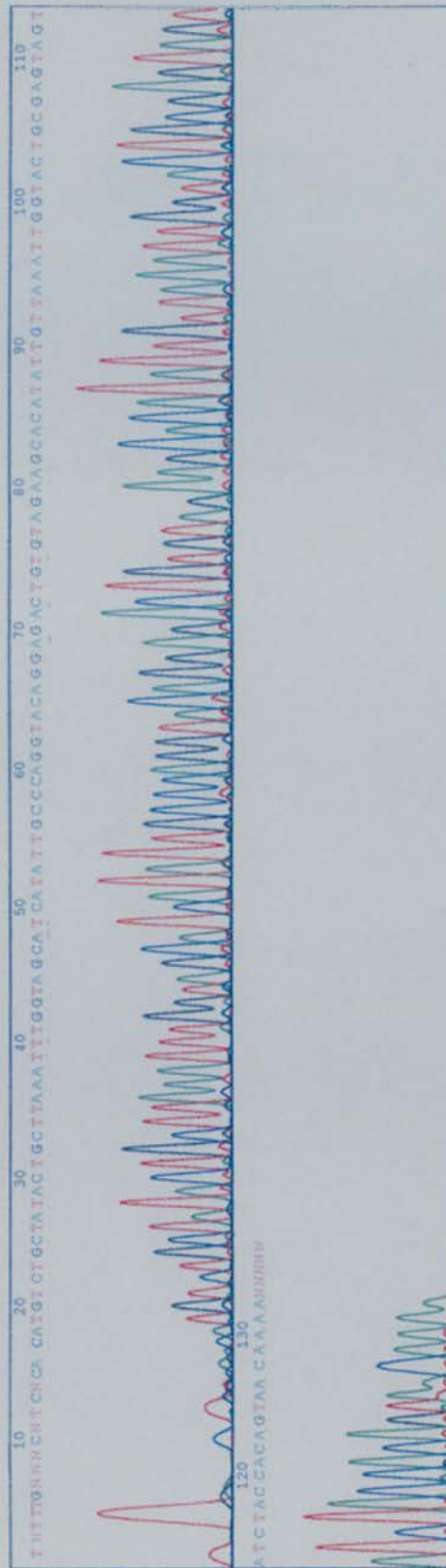
¹ HPV 70 = HPV 18 sub-group; related to HPV 39 (Longuet *et al.*, 1996)

² MM9 = HPV 73; high-risk genotype (Stewart *et al.*, 1996; Wallin *et al.*, 2000)

³ MM7 = HPV 83; low-risk genotype (Brown *et al.*, 1999)



GP5+

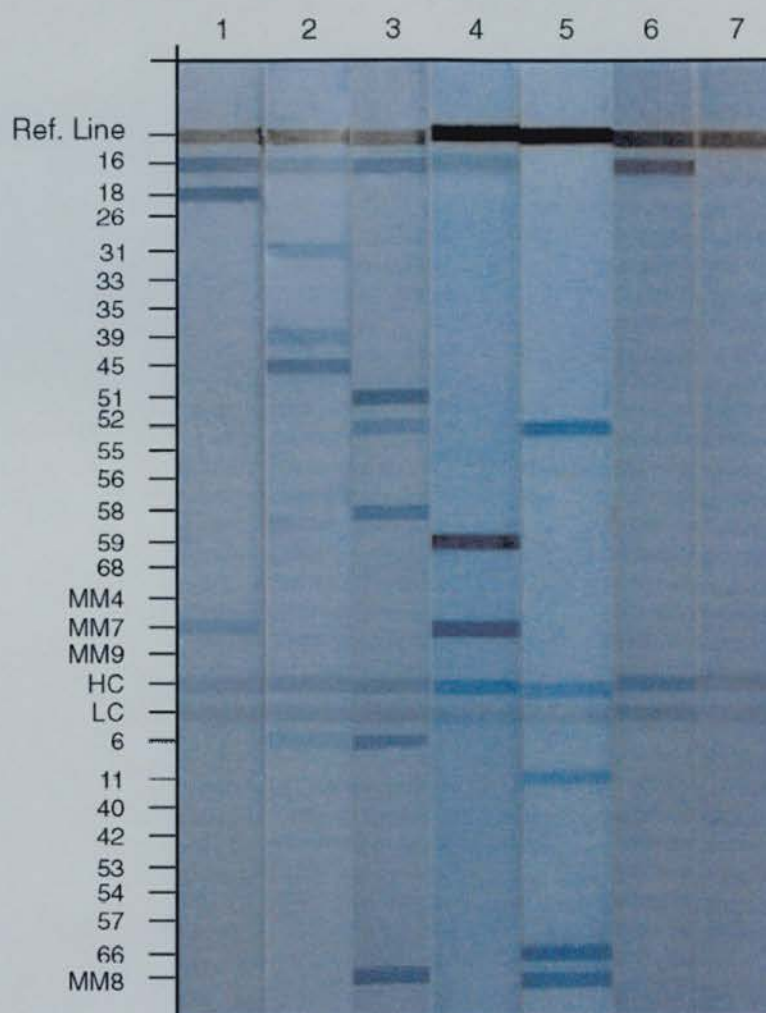


GP6+

Figure 5.1 HPV genotyping by direct DNA sequence analysis of GP5+/6+ PCR products. Cervical infection with a single HPV genotype identified as HPV18.

5.4.4 HPV genotyping using the RLBA

To identify the exact HPV genotypes present in 15 specimens (from 14 women) considered to contain more than one HPV genotype, RLBA testing was performed. Two samples had single HPV genotypes present; three samples had dual HPV infection; two samples had three different HPV genotypes present; three samples had four HPV types present and six different HPV genotypes were detected in one sample (Figure 5.4 and Table 5.5). Four specimens did not appear to contain any of the 27 genotypes detectable by RLBA, although both control lines were positive.



| Line No. | Patient Identifier | HR-HCAII result (RLU index) | GP5+/6+ PCR result | Direct DNA Sequencing | HPV genotypes detected by RLBA |
|----------|--------------------|-----------------------------|--------------------|-----------------------|--------------------------------|
| 1 | PS7 | Pos (169.24) | Pos | Mixed | HPV 16,18,MM7 |
| 2 | TJ1 | Pos (9.13) | Pos | Mixed | HPV 16,31,39,45 |
| 3 | BD1 | Pos (902.07) | Pos | Mixed | HPV 16,51,52,58,6,MM8 |
| 4 | JD2 | Neg (0.15) | Pos | Mixed | HPV 16,59,MM7 |
| 5 | CA8 | Neg (0.26) | Pos | Mixed | HPV 52, 11, 66, MM8 |
| 6 | SiHa DNA | NT | NT | NT | HPV16 |
| 7 | Neg Control | NT | NT | NT | None |

Figure 5.4 HPV genotyping using the Reverse Line Blot Assay showing five samples containing more than one HPV type

NT = not tested

| Lab No. | Cytology Result | No. of RLBA Bands | HPV Genotype(s) |
|-------------|-----------------|-------------------|--|
| GJB/98/0013 | mod | 6 | HPV 16 HPV 51 HPV 52 HPV 58 ⁴ HPV 6 MM8 ⁵ |
| GJB/98/0052 | mod | 2 | HPV 16 HPV 52 (<+) |
| GJB/98/0058 | mild | 3 | HPV 16 HPV 18 MM7 |
| GJB/98/0062 | U/S | 2 | HPV 18 HPV 54 |
| GJB/98/0078 | neg | 4 | HPV 16 HPV 31 HPV 39 HPV 45 |
| GJB/99/0128 | neg | 3 | HPV 16 (+) MM7 (+++) HPV 59 (+++) |
| GJB/99/0130 | neg | 4 | HPV 52 (++) HPV 66 (++) MM8 (++) HPV 11 (+) |
| GJB/99/0133 | neg | 4 | HPV 16 (+) HPV 58 (+++) HPV 52 (+) HPV 33 (+) |
| GJB/99/0139 | mild | 2 | HPV 18 (++) HPV 59 (+++) |
| GJB/98/0050 | neg | 0 | ND |
| GJB/99/0084 | neg | 1 | HPV 59 (+++) |
| GJB/99/0090 | neg | 0 | ND |
| GJB/99/0103 | neg | 1 | HPV 16 |
| GJB/99/0109 | neg | 0 | ND |
| GJB/99/0115 | mild | 0 | ND |

Table 5.5 HPV genotyping using the Reverse Line Blot Assay for 15 samples considered to contain more than one HPV type by direct DNA sequence analysis of the GP5+/6+ PCR product

ND = none detected

⁴ HPV 58 = HR-HPV genotype (Chan *et al.*, 1999)

⁵ MM8 = LR-HPV genotype (Meyer *et al.*, 1998b)

5.4.5 Detection of HPV DNA in follow-up cervical scrape specimens

Of the 20 women with more than one specimen, six (30%) appeared to be persistently infected with the same HPV type. Ten out of 14 women (70%), who had at least three cervical specimens taken at approximately six-monthly intervals were always HPV DNA positive but infected with different HPV types at different times.

Four HPV types whose oncogenicity still has to be determined or confirmed were found in fifteen specimens from 10 women. These were MM7 (now designated HPV83 (Brown *et al.*, 1999)), JC9710, CP6108 and CP8304 (Table 5.6). All four JC9710 isolates were HR-HCAII negative, GP5+/6+ PCR positive. CP6108 was also HR-HCAII negative, GP5+/6+ PCR positive although one sample did produce a weakly positive HR-HCAII result. On the other hand, CP8304 and MM7 genotypes were all HR-HCAII positive, despite these genotypes not being present in the HCAII high risk probe cocktail. Of these novel HPV genotypes, CP8304 was the only one to be found in association with cervical dysplasia. This genotype was detected in cervical scrapes from a 41-year old woman (Patient X). CP8304 was also associated with histologically confirmed CIN1 in this woman (Figure 5.5).

| HPV Genotype | No. of women | No. of isolates | Cytology Result | HR-HCAII Result | GP5+/6+ PCR Result |
|--------------|--------------|-----------------|-----------------|-----------------|--------------------|
| MM7 | 2 | 3 | U/S ×1 | Pos | Pos |
| | | | Neg ×2 | Pos ×2 | Pos ×2 |
| CP8304 | 4 | 6 | No smear ×1 | Pos | Pos |
| | | | Neg ×3 | Pos ×3 | Pos ×3 |
| | | | Mild ×1 | Pos | Pos |
| | | | Mod ×1 | Pos | Pos |
| CP6108 | 1 | 2 | Neg ×2 | Pos (1.43*) | Pos |
| | | | | Neg (0.63*) | Pos |
| JC9710 | 3 | 4 | Neg ×4 | Neg ×4 | Pos ×4 |
| TOTAL | 10 | 15 | | | |

Table 5.6 Four novel HPV genotypes identified by direct DNA sequencing of GP5+/6+ PCR products with the associated cytology and HPV testing results.

* HCAII RLU index value

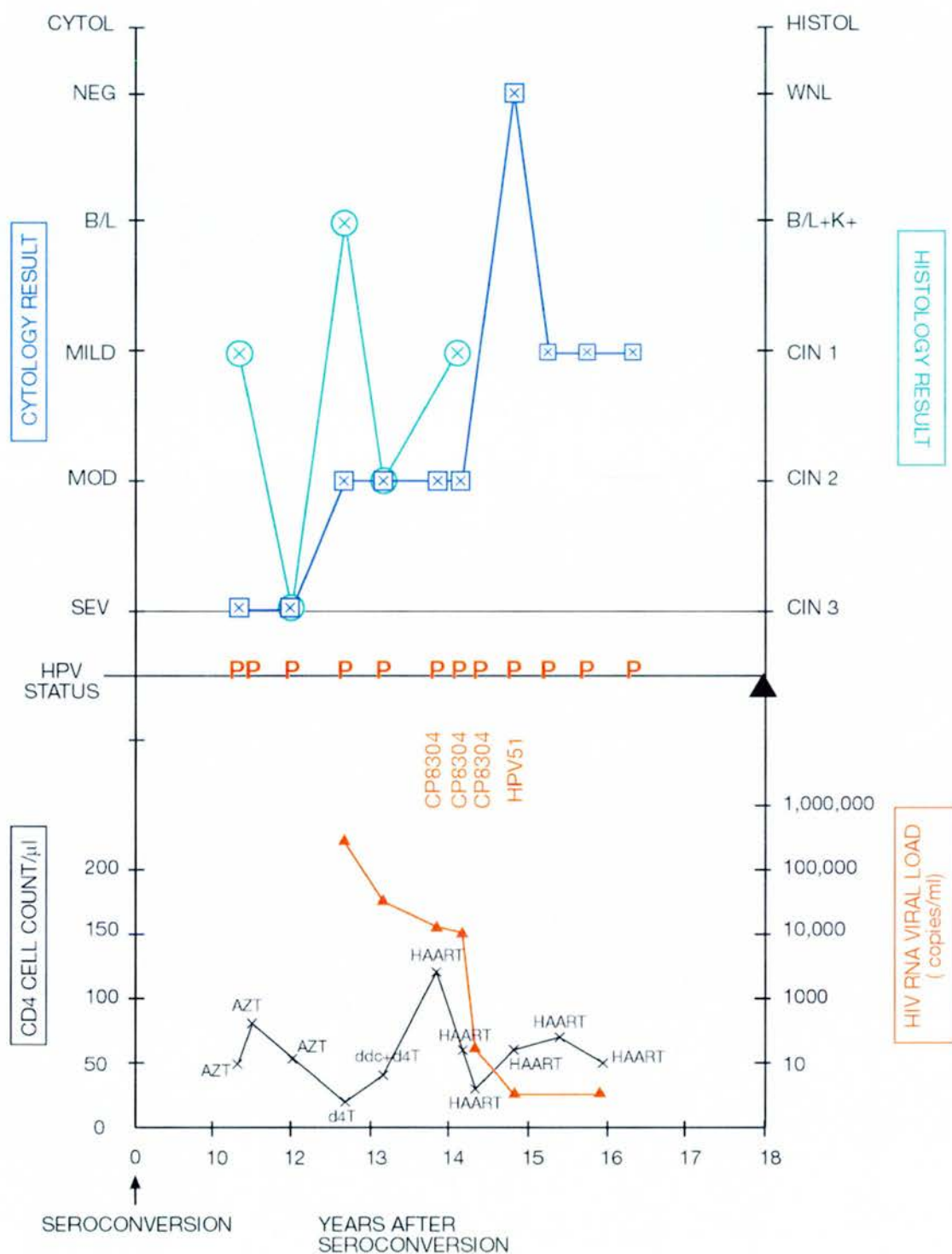


Figure 5.5 Summary of data collected from Patient X showing changes in cytology, histology, HPV status, CD4 cell count and HIV RNA viral load over time

P = positive

5.4.6 Influence of HIV-induced immunosuppression

At enrolment 23/114 women (20.2%) were receiving antiretroviral therapy (ART) when their first sample was taken (between May 1995 and November 2000). Four women were receiving one drug (AZT ×3; ddI ×1); ten women were receiving two drugs (AZT+3TC ×3; AZT+ddI ×1, AZT+ddC ×2; d4T+3TC ×2; d4T+ddC ×1; d4T+ddI ×1) and eight women were receiving HAART (unknown combination ×5; 3TC+NVP+RTN ×1; 3TC+NVP+AZT ×1, 3TC+NVP+d4T ×1). In addition, one woman was receiving her drug regime as part of a drugs trial. Some of the women in this group were receiving ART prior to July 1996 when HIV viral load testing became available and therefore nine samples collected had no corresponding HIV viral load result.

Of the 91 women who were not receiving ART when their first cervical specimen was collected, 59 had at least one other sample taken. 21/59 (35.6%) were receiving antiretroviral therapy when their last study specimen was collected: two women were receiving one drug (AZT ×1; 3TC ×1); four women were receiving two drugs (unknown combination ×1; 3TC+d4T ×2; ddI+d4T ×1) and fifteen women were receiving HAART (3 drugs ×12 ; 4 drugs ×1; 5 drugs ×2). 38/59 women were still not being treated with any antiretroviral drugs when their last cervical specimen was collected.

During the study period, 195 of an additional 269 specimens were obtained from women who had a corresponding HIV RNA viral load result. 87 specimens were collected from women who were not receiving antiretroviral drugs at the time of specimen collection. The median HIV RNA viral load result associated with these specimens was 18,873 copies/ml. A further 108 specimens were collected from women who were receiving ART of some kind at the time of specimen collection. Seven samples were obtained from women receiving one drug (median HIV RNA viral load = 20,786 copies/ml), 26 samples were obtained from women receiving two drugs (median HIV RNA viral load = 6,165 copies/ml) and 75 samples were obtained from women receiving HAART (median HIV RNA viral load = 140 copies/ml).

Of the 114 women who were receiving ART, 145 specimens were collected for cytological analysis from 52 women who had a corresponding HIV RNA viral load result and/or CD4 cell count. 18 women had one specimen collected, 13 women had two specimens collected, 8 women had three specimens collected and 12 women had between four and eight specimens collected. One woman had 12 specimens collected. Analysis of the cytology results in relation to immune status, as indicated by HIV RNA viral load and CD4 cell counts, can be seen in Tables 5.7 and 5.8 respectively. From Table 5.7, it can be seen that 45/124 specimens were obtained from women with HIV RNA viral load results >5000 copies/ml and 79 specimens were taken from women with HIV RNA viral loads <5000 copies/ml. 21 specimens had to be omitted from this analysis as they were obtained prior to the introduction of HIV viral load testing. Increased HIV RNA viral load appeared to be associated with a highly significant increased risk of abnormal cytology. 20/45 (44.4%) samples taken from women with HIV viral load results >5000 copies/ml were associated with some degree of cervical cytological abnormality (B/L or worse) compared with 16.4% (13/79) of samples taken from women with HIV viral load results <5000 copies/ml ($\chi^2 = 11.4993$ on 1 degree of freedom, $P < 0.001$).

| Cytology | HIV RNA VIRAL LOAD | | | | | TOTAL |
|---------------------------------------|--------------------|---------------------|------------------------|--------------------------|----------------------|------------|
| | <50 | 50–499 (HR-HPV+) | 500–4,999 (HR-HPV+) | 5000–49,999 (HR-HPV+) | >50,000 (HR-HPV+) | |
| Unknown | 0 | 0 | 0 | 0 | 1 | 1 |
| No smear | 0 | 5 (4) | 0 | 4 (4) | 1 (1) | 10 |
| Unsatisfactory (U/S) | 0 | 2 (1) | 0 | 2 | 1 | 5 |
| Negative (WNL) | 1 | 44 (14) | 14 (2, 1NT) | 10 (1) | 6 (1) | 75 |
| Borderline (B/L) | 0 | 3 (1) | 2 (2) | 2 (2) | 3 (3) | 10 |
| Mild dyskaryosis (Mild) | 0 | 5 (5) | 2 (1) | 2 (1) | 5 (5) | 14 |
| Moderate/severe dyskaryosis (Mod/Sev) | 0 | 1 (1) | 0 | 5 (5) | 3 (3) | 9 |
| TOTAL (HR-HPV+) | 1 | 60 (26) | 18 (5) | 25 (13) | 20 (13) | 124 |

Table 5.7 Relationship between HIV viral load and cytology in HIV–seropositive women

From Table 5.8 it can be seen that 61 (42.1%) of the 145 samples obtained from the same 52 women were associated with a CD4 count of less than 200 cells/ μ l. Of the 68 samples collected from women with a CD4 count above 200 cells/ μ l, only 20 samples (13.8%) were associated with CD4 counts of more than 500 cells/ μ l. HIV–infected women with abnormal smears showed lower CD4 lymphocyte counts ($\chi^2 = 5.4436$ on 1 degree of freedom, $P = 0.020$). Twenty–four out of 61 samples (39.3%) taken from women with CD4 counts <200 cells/ μ l were associated with cervical cytological abnormalities (B/L or worse), whereas 14/68 (20.5%) samples taken from women with CD4 counts of 200 cells/ μ l or more were found to have Pap smear abnormalities. HPV DNA detection was also found to be more common in samples taken from women with CD4 counts <200 cells/ μ l compared to women >200 cells/ μ l (57.4% and 39.7% respectively) ($\chi^2 = 4.0225$ on 1 degree of freedom, $P = 0.045$).

| Cytology | CD4 CELL COUNT | | | TOTAL |
|--|----------------------|---------------------|-----------------------|------------|
| | Unknown (HR-HPV+) | <200 (HR-HPV+) | 200–5000 (HR-HPV+) | |
| Unknown | 0 | 1 | 0 | 1 |
| No smear | 1 (1) | 7 (6) | 4 (4) | 12 |
| Unsatisfactory (U/S) | 1 | 3 | 2 (1) | 6 |
| Negative (WNL) | 11 (1, 1NT) | 26 (7) | 48 (10) | 85 |
| Borderline (B/L) | 3 (1) | 4 (3) | 7 (6) | 14 |
| Mild dyskaryosis (Mild) | 0 | 11 (10) | 5 (4) | 16 |
| Moderate/severe dyskaryosis (Mod/Sev) | 0 | 9 (9) | 2 (2) | 11 |
| TOTAL (HR-HPV+) | 16 (3) | 61 (35) | 68 (17) | 145 |

Table 5.8 Relationship between cytology and CD4 lymphocyte counts in HIV–seropositive women

5.5 Discussion

5.5.1 Cytological abnormalities in HIV–seropositive women

Abnormal cervical cytology was common in HIV–infected women from Lothian but the majority of abnormalities were low grade. Severe dyskaryosis was found in only 2.1% of satisfactory smears taken from the enrolled group. This supports a large US study where high grade changes were found in only 2.5% of HIV–infected women (Massad *et al.*, 1999). It has already been reported that high grade CIN (CIN 2/3) reached a peak in the Edinburgh HIV cohort in 1990–1992 and then dropped sharply. Low grade CIN (CIN 1) increased between 1987–1997 but also now appears to be decreasing (as shown in Figure 2; Cubie *et al.*, 2000) (Appendix 2). This had occurred well before the introduction of HAART and it is likely that regular monitoring and treatment of HPV–related cervical lesions has been of great significance in the clinical management of this group of HIV–seropositive women. The importance of adequate screening and treatment programmes for HIV–infected women to prevent progression to invasive disease has been highlighted by others (Vernon *et al.*, 1995; Goodman *et al.*, 1999).

5.5.2 HPV testing methods

5.5.2.1 GP5+/6+ PCR and HCAII

On comparison of GP5+/6+ PCR and HCAII testing methods for the detection of HPV DNA, the PCR–based method resulted in a higher HPV detection rate compared to HR–HCAII. Unlike HCAII, the GP5+/6+ consensus primer PCR method can detect high–risk, low–risk and novel HPV genotypes. However, PCR amplification of certain HPV types may differ depending on the primer set chosen (Karlsen *et al.*, 1996; Qu *et al.*, 1997; Baay *et al.*, 1996). For example, GP5+/6+ primers have been shown to be less effective at detecting multiple infections compared to other primer systems available (Qu *et al.*, 1997). Improved HPV detection in HIV–seropositive women using MY09/11 primers has been shown by Uberti–Foppa *et al.*, (1998), where HPV was detected by HCAII in 66% of cases but MY09/11 PCR was positive in 91%.

In this study, using HCAII alone would have missed 27 cases of HPV infection, mostly due to LR-HPV genotypes. In contrast, nine specimens were HR-HCAII positive but gave negative results upon GP5+/6+ PCR testing. These are most likely false positive HCAII results since the RLU ratios of all except one sample were less than 3. It is likely that upon repeat testing these samples would be HR-HCAII negative. The HCAII results obtained were not used quantitatively although it has been shown that HIV-seropositive women with normal cytology and HPV positivity by HR-HCAII have more than seven times the amount of HPV DNA compared to HPV-positive, HIV-seronegative women (Womack *et al.*, 2000) and high viral load but not viral risk category has been associated with disease persistence among HIV-seropositive women (Ahdieh *et al.*, 2001). Quantitative measurements of HPV DNA by HR-HCAII have also been strongly associated with high-grade CIN in HIV-seropositive women (Cohn *et al.*, 2001) and some correlation has been shown between HCAII RLU indices and the degree of squamous dyskaryosis in the Edinburgh HIV-infected population (as shown in Table 2, Cubie *et al.*, 2000 (Appendix 3). Sequencing data from the study reported here, showed that the high-risk HCAII probe pool mix allowed detection by cross-hybridisation to additional HPV genotypes. For example, all samples containing CP8304 and MM7 were HR-HCAII positive, and GP5+/6+ PCR positive. The type specificity of HCAI has been investigated by Kónya *et al.*, (2000) who concluded that several HPVs including HPV53, 58, 62, 66, CP8304 and MM4 were detectable by the this test despite the lack of specific probes for these types in the probe cocktail. Using HCAII, detection of HPV53, 66, 67, 73, CP6108 and CP8061 has been reported (Peyton *et al.*, 1998) and cross-reactivity of some HR-HPV types with LR-HPV genotypes has also be found (Meyer *et al.*, 2001a).

5.5.2.2 Direct DNA sequencing of GP5+/6+ PCR products

Using a small aliquot of the GP5+/6+ PCR product as a template for direct DNA sequencing proved to be a powerful method for HPV genotyping. It is particularly suited to the identification of a wide range of genotypes, including rare, novel or uncharacterised types, that may not be identified by other methods (Bernard *et al.*,

1994). Indeed, direct sequencing of PCR products has been used in other studies for identification of novel HPV genotypes JC9710 (Feoli-Fonseca *et al.*, 1998a) and JC9813 (Feoli-Fonseca *et al.*, 1998b) from HIV-positive women. It is therefore possible that additional new HPV genotypes may be discovered in this population using PCR-direct sequencing or rare types may be further characterised in terms of oncogenicity. However, the drawbacks of DNA sequencing included difficulty resolving low quality sequence data from true multiple infection. In such cases, manual interpretation of sequence data was sometimes necessary to assign an HPV type. Since the L1 region is highly conserved between different HPV types and the PCR product sequenced was only 150bp in size, sometimes it was difficult to obtain sufficient sequence homology to confidently assign a single HPV genotype and therefore these were considered true multiple infections. It is known that the presence of excess primer can interfere with the sequencing reaction but attempts to improve this were unsuccessful. Ambiguous sequence results could have been resolved by reamplification and cloning but this was not possible during the current study. A software database has now been created by Feoli-Fonseca and colleagues (1999) to address the diagnostic problem of overlapping sequences (multiple peaks at a given nucleotide position) that signify infection with more than one HPV type.

5.5.3 Novel HPVs and multiple HPV infection

HPVs 16 and 18 are documented to be the most prevalent genotypes in both HIV-infected and non-infected women (Capiello *et al.*, 1997; Chopra and Tying, 1997). Although HPV 16 and HPV 18 were most commonly detected in the current study (29.2% prevalence for both), a total of 22 different HPV genotypes were found in single infections. These findings are comparable to other studies where a variety of HPV genotypes have been identified in HIV infected women, including novel HPV types, which may or may not be oncogenic (Cappiello *et al.*, 1997; Meyer *et al.*, 1998; Gonçalves *et al.*, 1999, Slavinsky *et al.*, 2000). Indeed, new HPV types have been identified in oral warts with atypia from HIV-infected patients (Völter *et al.*, 1996). However, only the novel HPV type CP8304 was associated with cervical dyskaryosis and histologically confirmed cervical disease. This genotype, which is closely related to HPV62 (84.5%), a low-risk HPV genotype, was first identified in

women from New Mexico who had normal cervical cytology (Peyton and Wheeler, 1994). More recently, Meyer *et al.*, (2001b) have concluded that CP8304 is probably not associated with a high risk of malignant progression since this viral type has never been detected in cervical cancer specimens. HPV sequences CP8304, CP6108 and MM8 and HPV types 61 and 62 are all members of the A3 group (internet citation <http://hpv-web.lanl.gov> and Figure 1.1).

PCR-based assays have been used by others to show that HIV seropositive women harbour a higher proportion of multiple HPV genotypes in the cervix compared to HIV seronegative women (Eckert *et al.*, 1999; Torrisi *et al.*, 2000). In the study reported here, direct DNA sequence analysis of GP5+/6+ PCR products indicated that 15 samples contained more than one HPV genotype. The RLBA proved a useful tool for determining the exact HPV types present in samples containing more than one HPV genotype. Using this method, 4 of 15 samples were found to contain between four and six different HPVs. The absence of HPV detection in four samples does not mean that HPV is not present. Such results may have arisen due to either a false positive PCR result or due to the presence of other HPV genotype(s) that cannot be detected by this method due to the design of the probes used. Due to the lack of type-specific probes for rare or novel HPVs, the prevalence of such HPVs in particular may not have been fully realised (Ong *et al.*, 1994; Peyton *et al.*, 1994). The challenge of future studies will be to show whether particular novel HPV genotypes are clinically relevant. If so, then the range of specific probes included in HPV assays will need to be expanded.

Findings from the study reported here also show that the HPV genotypes harboured in the cervix of HIV-infected women do not always remain the same over time. In a study by Miotti and colleagues (1996) only 62.5% women with HPV detected at two visits had the same HPV type. Although changes in lifestyle and sexual practice could influence the acquisition of HPV types, it has been suggested that 'epidemiological waves' of HPV types could be likely (Jin *et al.*, 2000). If the HPV genotypes do not remain the same then single time point HPV testing may considerably underestimate both the prevalence of HPV infection and the genotypes

associated with cervical neoplasia. Therefore care must also be taken about the use of the term 'persistent infection'.

In the study reported here, 70% of women who had at least three cervical specimens taken were shown to be HPV DNA positive in every sample, although this was not necessarily associated with the progression of cervical disease. These findings support the results of a longitudinal study of HPV carriage in HIV-infected and non-infected women, (Minkoff *et al.*, 1998) that concluded that HIV seropositive women were more likely to have newly detectable oncogenic HPV at follow-up and were more likely to have HR-HPV regularly detected. In a study by Ahdieh *et al.*, (2000), where study participants had a median of six visits, 59.3% of moderately immunosuppressed women and 83.9% of severely immunosuppressed women were HPV positive in all samples. The increased prevalence and persistence of HPV in HIV-infected women is most likely due to the inability of the hosts depleted immune system to clear the viral infection.

5.5.4 The effects of HIV-induced immunosuppression

In an African study, 70% of HIV-infected women with genital HPV infection appeared to have a persistent infection which was still present 12 months later (Miotti *et al.*, 1996). Indeed in women with CD4 counts <200 cells/ μ l, HPV infection is unlikely to be eliminated, as shown in the study by Ahdieh and colleagues (2000) where only 16.1% of severely immunocompromised women cleared their HPV infection. The influence of immunosuppression was also highlighted by Six *et al.*, (1998) where CD4 counts of <500 cells/ μ l were associated with a higher incidence of cervical abnormalities compared to women with CD4 counts above 500 cells/ μ l. In fact, no cervical disease progression was observed in HIV-seronegative women or HIV-seropositive women whose CD4 count remained above 500 cells/ μ l. In addition, a 2-year follow-up study by Sun *et al.*, (1997) showed the prevalence of HPV in HIV-infected women with CD4 counts less than 500 cells/ μ l was 95% compared to 74% in women with CD4 counts of 500 cells/ μ l or above. In the study reported here, women with CD4 counts less than 200 cells/ μ l were at greatest risk of cervical disease and were found to harbour HPV DNA more frequently compared to

women with CD4 counts greater than 200 cells/ μ l. These findings thus support suggestions that a woman with a history of abnormal cytology and a CD4 count less than 200 cells/ μ l should be frequently screened (Goodman *et al.*, 1999).

The effect of HAART on both cervical HIV and HPV viral load remains unclear. The risk of developing squamous intraepithelial neoplastic lesions has been shown to be higher in women not receiving antiretroviral therapy (Delmas *et al.*, 2000) and elevated HIV RNA levels of >10,000 copies/ml has been shown to be significantly associated with abnormal cytology (Luque *et al.*, 1999). In the study reported here, women receiving antiretroviral therapy with HIV RNA viral load results of >5000 copies/ml appeared to be at greatest risk of cervical disease.

Heard and colleagues (1998) showed that HAART reduced the prevalence of cervical lesions during follow-up despite the presence of persistent HPV infection (although the numbers employed were too small to assess the incidence of cervical neoplasia in relation to the type of antiretroviral therapy being administered). In an Italian study by Lillo *et al.*, (2001), HAART had no significant effect on the prevalence of cervical lesions or HR-HPV. Indeed the incidence of invasive cervical cancer in Italy does not appear to have declined, despite the introduction of HAART and an associated decrease in prevalence of other AIDS-defining diseases since 1996 (Dorrucchi *et al.*, 2001). Many women in the Edinburgh HIV cohort have been followed up after 1996 when HAART and HIV viral load testing became available thus providing an ideal population for further study on the effect of antiretroviral therapies on HIV RNA viral load, HPV DNA detection and the subsequent occurrence of cervical neoplasia.

5.6 Conclusion

It is apparent that the role of HPV in HIV-infected women is multi-faceted involving persistent HPV infection, multiple HPV types, rare or novel genotypes and possibly HPV variants. Although persistent HPV infection was common in the Edinburgh cohort, this was not necessarily associated with progression of cervical disease. However, the risk of squamous dyskaryosis was increased if CD4 lymphocyte counts were less than 200 cells/ μ l or the HIV RNA viral load was greater than 5000 copies/ml. These findings support suggestions that HIV-infected women should be screened more frequently. It remains to be seen if HAART will improve local immune function and reduce the prevalence of cervical neoplasia and HPV persistence.

Findings from this study, and others, suggest that the true prevalence of cervical HPV infection in HIV-seropositive women has been underestimated. Some previous studies have adopted screening methods that are not capable of detecting all HPVs and do not differentiate between individual HPV genotypes. GP5+/6+ PCR resulted in a higher HPV detection rate than HCAII (59% vs. 47%), as this method will also detect low-risk and rare HPV genotypes. Direct DNA sequence analysis of GP5+/6+ PCR products provided very important information regarding the distribution of HPV genotypes in this immunocompromised population. A total of 22 different HPV types were identified in single infections including some rare or novel HPV types which, to date, have not been fully characterised. Of these novel HPV genotypes, only CP8304 was associated with cervical dysplasia. Some cross-reactivity with the high-risk probe cocktail of HCAII was seen with other HPV types. Additionally, the HPV genotypes harboured in the cervix do not always remain the same over time. In this study, 12 out of 44 (27.3%) women who had samples tested by direct sequence analysis and RLBA had infection with more than one HPV genotype. Up to six different HPVs were detected by the RLBA from DNA extracted from a single cervical specimen. Ultimately, understanding the distribution and oncogenicity of all HPV types is important for clinical management of patients and vaccine development.

By the end of 2000, there were 759 known HIV-infected women in Scotland (www.nhsis.co.uk/scieh/infectious/aidshiv/inf aids) but there are no national guidelines for cervical screening or clinical management of cervical neoplasia in this population (Vonau *et al.*, 1999). HPV testing may not be useful for directing clinical management of HIV-seropositive women since the prevalence of HPV infection is high in this population (as shown by the 25.3% of samples from women without any cytological abnormality that were found to harbour HPV in the cervix) but this does not appear to be associated with a higher incidence of CIN. However, HPV genotyping may be a useful tool for identification of those women at most risk of disease progression.

Although management of HIV-infected women within a cervical screening programme remains controversial, it appears that cervical disease progression in the Edinburgh HIV-infected cohort is contained by regular examination, early detection of precancerous lesions, effective treatment and thorough follow-up. However, the interactions between HIV, HPV and the development of cervical disease warrants further investigation in longitudinal studies over at least 10 years. The Edinburgh cohort will be followed as part of the 'Management of Cervical Screening in HIV-1' (MACH-1) study that involves collecting data from various European centres to determine the best cervical screening and treatment programme for the prevention of cervical disease in HIV-infected women. Since an American study showed black and young hispanic HIV-infected women to be at most risk of invasive cervical cancer (Chin *et al.*, 1998), it is important that such guidelines are introduced internationally so that access to medical care facilities is made routinely available to all HIV-infected women regardless of ethnic background or social status.

CHAPTER 6

HPV IN WOMEN WITH BORDERLINE CYTOLOGICAL ABNORMALITIES

6.1 Introduction

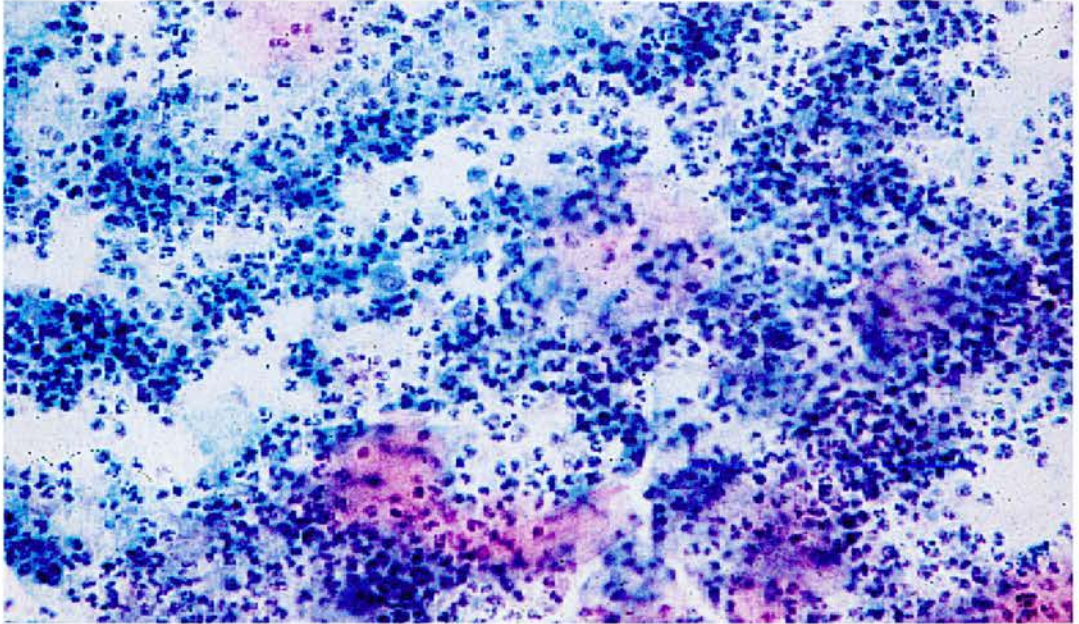
Despite a reduction in the cervical cancer incidence rate due to Pap smear screening, the limitations of this method are now well recognised. In a study by the US Agency for Health Care Policy and Research, the sensitivity of the Pap smear ranged from 29% to 56% (mean 49%) and the specificity was estimated at between 97–100% (AHCPR Publication, 2000). Between 67% and 90% of all false negative Pap smears occur due to sampling error (Gay *et al.*, 1985; Yeoh and Chan, 1997). Depending on the sampling device used, most of the cells removed from the cervix are not transferred onto the glass slide and are discarded along with the sampler (6.5% at best and 62.5% at worst in a study by Hutchinson *et al.*, 1994). For a variety of reasons as many as 15% of smears are reported as inadequate for laboratory diagnosis (McGoogan and Reith, 1996). The main reasons for this are: insufficient cells are present; inflammation and blood can often obscure cells; cells can be overlapping; and air-drying artefacts are common. In addition, up to 20% of Pap smears are reported as atypical (B/L nuclear changes or ASCUS) (McGoogan and Reith, 1996). In the USA, ASCUS Pap diagnoses (approximates to B/L nuclear changes) constitute approximately 2 million cytology reports (Manos *et al.*, 1999) and B/L smear reports account for approximately 5% of the annual 90,000 smears workload in South-east Scotland (Dr McGoogan, personal communication). Moreover, only 5–10% of women with ASCUS Pap smear reports harbour serious cervical disease but more than one third of high-grade cervical lesions in screening populations have been identified from further investigation following ASCUS Pap results (Manos *et al.*, 1999; Kinney *et al.*, 1998). Although immediate colposcopic examination of women with minimally abnormal smears is considered the safest option (Soutter and Fletcher, 1994), this is not practical due to the resources

available. Currently, management of women with borderline smear reports leads to recall for a repeat Pap smear in six months and then referral for colposcopic examination to identify underlying CIN and treatment if necessary. Unless two subsequent negative smear reports at six monthly intervals are obtained the patient will not be safely returned to routine recall. Since only a small percentage of these women will have clinically significant disease unnecessary pressure is imposed on colposcopy clinics and many women are subject to an inessential, psychologically stressful procedure. With the introduction of LBC, where the entire cervical specimen is collected into a vial of preservative solution, a more accurate assessment of cervical dyskaryosis is now possible. Automated technology is used to make monolayer preparations, free from blood and mucus, which can be screened more effectively and efficiently than conventional Pap smears (Figure 6.1).

Since the association between HR-HPV types and the development and progression of CIN and cervical cancer has been clearly demonstrated, HR-HPV testing has been suggested as a strategy to improve the management of women with low-grade cytological abnormalities (Cox *et al.*, 1999). HR-HPV prevalence precedes abnormal cytology in women who develop cervical cancer (Zielinski *et al.*, 2000b) and HPV positivity has been associated with an 8-fold increased risk of histological confirmation of cervical neoplasia (Cox *et al.*, 1995) whereas HPV negative women are more likely to have no visible lesion at colposcopy or to have a lesion that will be diagnosed as negative after biopsy. By introducing HPV testing, women with B/L smears may be more effectively managed into one of two groups: continued cytological follow-up or immediate referral to colposcopy depending on their HR-HPV status.

The aim of the work presented in this chapter was to compare the cytologic diagnoses of ThinPrep® slides and conventional Pap smears and to assess whether HR-HPV testing using HCAII would be a valuable triage method for women with borderline cytological abnormalities in order to allow better identification and management of women whose cervical lesions are likely to persist.

(a)



(b)

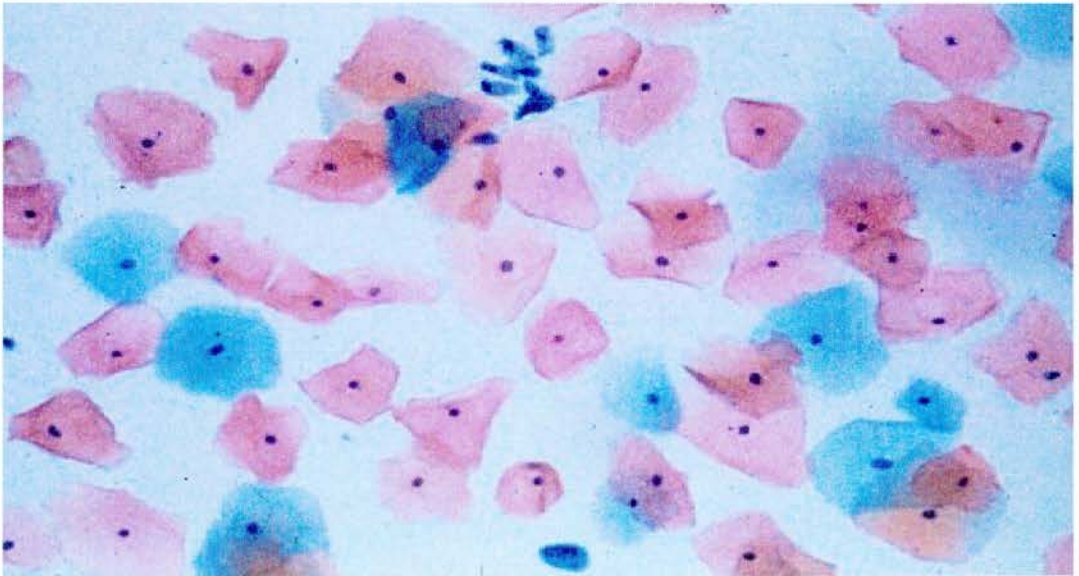


Figure 6.1 Comparison between conventional Pap smears and ThinPrep® slide preparations

(a) A manually prepared, conventional Pap smear designated unsatisfactory for assessment since the smear is obscured by inflammation, preventing interpretation of 50–75% of the epithelial cells.

(b) A ThinPrep® slide prepared using the TP2000 automated slide processor. The cells are displayed as a monolayer within a 20mm diameter circle on the glass slide.

6.2 Methods

6.2.1 Study population

Between March 1998 and February 2000, 903 women, aged 18–69 years, providing routine or follow-up cervical smears in a primary care setting were recruited. 246 women (mean age = 34.2 years) who were attending for an early repeat smear following a borderline (B/L) or mildly dyskaryotic smear were targeted (Appendix 2). This smear is indicated as the index smear. 206 women recruited had a previous B/L abnormality and 40 women had a previous mild abnormality (including 1 with an interim negative smear). These 246 cases were considered the *study set*. Additionally, samples were collected from other women, who had provided consent and were undergoing routine screening at the same clinics on the same day. Once the cytology result was known, women who were the same age and had no history of cervical abnormality were then matched to individual study cases, thus forming an age-matched *control set*. Wherever possible, the criterion used to match each control was women attending the same practice who had no more than five years age difference with the study case. If this was not possible, a woman with an age difference of one year from any practice was used. Finally, women with an age difference of five years from any practice were assigned as controls if necessary. 220 controls are included in this analysis. The remaining 437 samples were collected from other women who attended these clinics on the same day and who were not assigned to either the study or control set.

| | | |
|--------------|--|------------|
| Study Set | Women recruited with a previous B/L smear report | 206 |
| | Women with a previous mildly dyskaryotic smear report | 40 |
| Control Set | Women (age-matched) with no previous cytological abnormalities | 220 |
| ‘Others’ | Women not assigned to either the study or control set | 437 |
| TOTAL | | 903 |

Table 6.1 Specimens collected from women attending primary care facilities for routine or follow up cervical smears

6.2.2 Specimen collection and processing

This was a 'split sample' study: cells were collected from the cervix using a plastic 'broom-like' sampling device (Cervex-brush®, Rovers Medical Devices B.V., The Netherlands). A routine Pap smear was prepared first in the standard way before the residual material on the sampler was rinsed vigorously in 20mls of PreservCyt® solution. The sampling device was then discarded. ThinPrep® slides were made as described in Section 2.1.

6.2.3 HPV DNA testing

After TP monolayer preparation, specimens were sent by van to the HPV testing laboratory at the RCVL. Specimens were stored at ambient temperature until specimen processing was performed. Specimen processing was carried out within 21 days after sample collection, as recommended by the manufacturer. In a small number of cases this was not possible and the samples were kept at 4°C until processed. 4ml of PreservCyt® fluid was used for the Digene Sample Conversion protocol to make the specimen suitable for HCAII testing (Section 2.7). The residual fluid present was processed for DNA extraction and stored at -20°C until required. DNA extraction was performed using QIAGEN spin columns (Section 2.5.2). To increase sample throughput, DNA from some specimens was extracted using the QIAGEN QIAVac® 24 procedure (Section 2.5.3) rather than the totally manual protocol. PCR testing was performed using conventional GP5+/6+ PCR (Section 2.8.1).

If less than 4ml of PreservCyt® fluid was present in the specimen vial, the exact volume was noted and the cells pelleted directly. After removal of the supernatant, the dry pellet was stored at -20°C.

6.2.4 Cytology reporting

The conventional Pap smears and TP monolayers were examined and reported independently. Pap smears were screened by trained cytotechnologists, confirmed by consultant pathologists and reported as the diagnostic smear using the NHS CSP national guidelines. Two consultant pathologists (Dr E McGoogan and Dr A Williams) classified the TP smears. The TP cytologic diagnoses were translated to

the Bethesda system for cervical/vaginal diagnostic reporting to allow results from the different cervical cytology methods to be easily distinguished. There are however important differences between these nomenclature schemes. Firstly, B/L nuclear change includes visible HPV cytopathic effect (i.e. koilocyte development) whereas ASCUS does not include HPV changes. Secondly, in the UK, an unsatisfactory diagnosis is applied when less than 33% of the slide ($40 \times 22\text{mm}$ coverslip) is covered with squamous epithelial cells (British Society for Clinical Cytology, 1990) whereas in the USA, smears are classified as inadequate if less than 10% of the slide ($60 \times 24\text{mm}$ coverslip) is covered with cells.

The TP monolayer results were used for research purposes only and were not given to the clinicians. Thus clinical management was determined on the basis of the conventional smear result only. Likewise, HPV test results were not used to influence the management of patients. Ethical permission for this study was received from Lothian Health (LREC Number 1702/97/4/16).

6.2.5 Patient follow up

Follow-up information for women recruited to the study was found using the pathology department computer system. Management of cervical abnormalities was performed in accordance with NHS CSP national guidelines. No LBC specimens were collected during the follow-up period. Women with U/S smears were recalled within one month for a repeat smear. Women with B/L reports were re-examined in 3–6 months by repeat Pap smear. Women with normal results were followed up 12–36 months later with repeat Pap smears, depending on their previous cervical screening history. If high-grade cervical lesions were detected, patients were referred for colposcopy, biopsy with histological examination and treatment where appropriate.

6.3 RESULTS

6.3.1 Comparison of ThinPrep® and conventional Pap Smear cytology data for all study participants

103 TP results were not available to allow comparative analysis. Analysis of the cytology results obtained for the remaining 800 TP and conventional smears can be seen in Table 6.2. Sixteen slides diagnosed as inadequate by TP and 58 diagnosed as U/S by Pap smear were omitted from further analysis. Of the 726 satisfactory slides, 641 (88.3%) showed complete diagnostic agreement between TP and Pap smear results. 51 of the 90 discrepant cases (56.7%) occurred due to the detection of a more severe abnormality by TP analysis. These included 11 cases that were reported as normal by conventional Pap smear but mild dyskaryosis (equivalent of LSIL) was detected by the TP smear and 3 cases that appeared normal by Pap smear screening but showed moderate or severe dyskaryosis (equivalent of HSIL) on the TP slide.

Conventional Pap smears showed a more severe abnormality in 39 cases (43.3%). These included 38 cases where a borderline (equivalent of ASCUS) or mildly dyskaryotic (equivalent of LSIL) smear diagnosis had been reported by conventional Pap smear but designated WNL by TP analysis. One case was diagnosed as moderate/severe dyskaryosis by conventional Pap smear but was reported as LSIL by TP analysis.

Despite the use of 'left-over' material for this split sample study, TP analysis reduced the number of inadequate smear results compared to the conventional Pap smear (from 58 (7.3%) to 16 (2%)). The inadequate TP smear reports largely resulted from insufficient epithelial cells present on the slide (data not shown). Likewise TP reduced the number of borderline (equivalent of ASCUS) diagnoses from 63 (7.9%) to 34 (4.2%). In addition, TP analysis identified eight more cases of low-grade cervical abnormality and 13 additional cases of high-grade neoplasia. Fifteen patients of 673 with inadequate or negative reports by the Pap smear had a significant abnormality (LSIL or worse) detected by TP analysis: four out of 12 women with low-grade smear abnormalities detected by TP and one of three women with high-grade dyskaryosis detected by TP had subsequent conventional Pap smears during follow-up. All were considered to be WNL at this stage.

| ThinPre® slide diagnosis | Conventional Pap smear diagnosis | | | | | TOTAL |
|--------------------------------|----------------------------------|-----|-----------------|----------------|----------------|------------|
| | U/S | NEG | B/L | Mild | Mod or Sev | |
| INAD | 5 | 9 | 2 | 0 | 0 | 16 |
| WNL | 51 | 576 | 32 [†] | 6 [†] | 0 | 665 |
| ASCUS | 1 | 16* | 17 | 0 | 0 | 34 |
| LSIL | 1 | 11* | 10* | 25 | 1 [†] | 48 |
| HSIL | 0 | 3* | 2* | 9* | 23 | 37 |
| TOTAL | 58 | 615 | 63 | 40 | 24 | 800 |

Table 6.2 Cytology results for 800 ThinPrep® slides and conventional Pap smears

Conventional Pap smears were classified using the Richardt classification system: U/S = unsatisfactory for assessment; Neg = negative; B/L = borderline nuclear changes; mild = mild dyskaryosis; mod = moderate dyskaryosis; sev = severe dyskaryosis.

ThinPrep® slides were classified using the Bethesda classification system: INAD = inadequate for diagnosis; WNL = within normal limits; ASCUS = atypical squamous epithelial cells of undetermined significance; LSIL = low-grade squamous intraepithelial lesion; HSIL = high-grade squamous intraepithelial lesion.

[†] Detection of a more severe cytological abnormality using the Pap smear

* Detection of a more severe cytological abnormality using TP analysis

6.3.2 Detection of HPV DNA in cervical samples from all study participants using HCAII

Sixty-three (7%) of 903 specimens were not included for HPV testing. This included 55 specimens that had insufficient PreservCyt® volume (<4mls) and therefore did not meet the manufacturer's guidelines for Cytoc specimen conversion. 745 of 840 satisfactory specimens had results available for both cytology methods and HR-HPV testing by HCAII (Table 6.3). The overall HR-HPV prevalence was 29.1% (217/745).

6.3.3 Comparison between HCAII and PCR for the detection of HPV DNA in cervical samples from all study participants

387 out of 840 satisfactory specimens were also tested using conventional GP5+/6+ PCR. There was overall agreement between GP5+/6+ PCR and HCAII for the detection of HPV DNA in 87.6% of the samples tested (Table 6.4).

It was only possible to test a few of these samples by LightCycler GP5+/6+ PCR (LC PCR). This included 37 of the 116 specimens that had tested HPV positive using HCAII and conventional GP5+/6+ PCR. 34 results were confirmed by LC GP5+/6+ PCR (Figure 6.2). The remaining three LC PCR negative cases were found to be false negative LC PCR results since HPV 31, HPV 54 and HPV 59 were detected by direct DNA sequencing of the conventional GP5+/6+ PCR product. Fifteen of 27 samples that gave HCAII positive results and conventional PCR negative results were also tested. 13/15 samples that had tested HPV positive using HR-HCAII were found to be HPV negative by both conventional and real-time GP5+/6+ PCR methods. These most likely indicate false positive HR-HCAII results since 5 out of 13 specimens gave HCAII ratios near to the manufacturers' cut-off highlighting that caution must be applied when interpreting low-level HCAII positive results. Lack of resources prevented further LC PCR testing in this group.

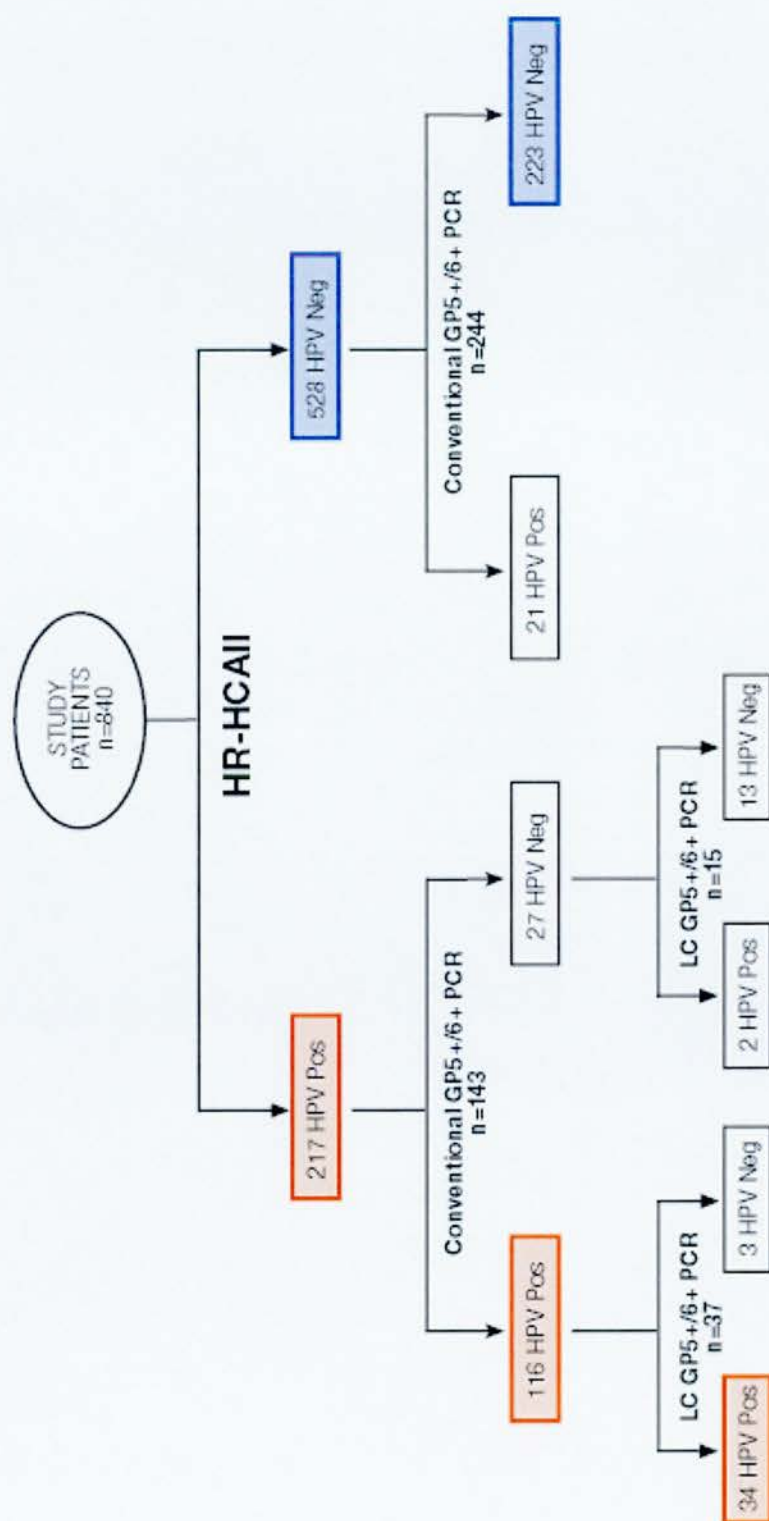


Figure 6.2 Summary of HPV testing using HCAII, conventional GP5+/6+ PCR and LightCycler GP5+/6+ PCR in satisfactory cytology specimens collected from the entire study cohort

| Cytology | No. of Pap smears | HPV Pos | No of TP smears | HPV Pos |
|--------------------|----------------------------------|--------------------|--------------------------------|--------------------|
| U/S or INAD | 57 | 12 (21%) | 15 | 3 (20%) |
| Neg | 567 | 120 (21.2%) | 614 | 121 (19.7%) |
| B/L or ASCUS | 58 | 28 (48.3%) | 33 | 18 (54.5%) |
| Mild or LSIL | 39 | 34 (87.2%) | 46 | 39 (84.7%) |
| Mod/Sev or HSIL | 24 | 23 (95.8%) | 37 | 36 (97.3%) |
| TOTAL | 745 | 217 (29.1%) | 745 | 217 (29.1%) |

Table 6.3 HR–HPV results by HCAII in relation to the conventional Pap smear and ThinPrep® cytology results for 745 satisfactory specimens collected with all data available

| | | GP5+/6+ Conventional PCR | | TOTAL |
|-----------------|------------|---------------------------------|------------|--------------|
| | | POS | NEG | |
| HR–HCAII | POS | 116 | 27 | 143 |
| | NEG | 21 | 223 | 244 |
| TOTAL | | 137 | 250 | 387 |

Table 6.4 Comparison of HPV results by HCAII and GP5+/6+ PCR for the 387 samples that were tested using both methods

6.3.4 Detection of HPV DNA in cervical samples from women with previous borderline smears

Two samples from women assigned to the *study set* were not tested by HR-HCAII. As shown in Table 6.5, HR-HPV DNA was detected by HCAII in 44.7% of smears obtained from the remaining 244 women in the *study set* (i.e. those with a previous borderline or low-grade cervical abnormality). 100% of samples associated with smears showing high-grade dyskaryosis (the equivalent of HSIL) by either TP or conventional Pap smear were HR-HPV positive. Approximately 80% of samples associated with low-grade cytological abnormalities (equivalent of LSIL) were HR-HPV positive by HCAII. 50% and 61% of women whose repeat smear showed B/L abnormalities by conventional Pap and TP cytology respectively were found to be HR-HPV positive.

| | STUDY SET (n=246; 2NT) | | | | | |
|-----------------|------------------------|---------|-------|-----------------|---------|-------|
| | Conventional Pap smear | | | ThinPrep® smear | | |
| | n | HPV Pos | % | n | HPV Pos | % |
| U/S or INAD | 14 | 3 | 21.4 | 5 | 1 | 20.0 |
| Neg | 159 | 55 | 35.0 | 173 | 53 | 31.0 |
| | (2NT) | | | (2NT) | | |
| B/L or ASCUS | 35 | 17 | 50.0 | 18 | 11 | 61.1 |
| Mild or LSIL | 26 | 22 | 84.6 | 30 | 24 | 80.0 |
| Mod/Sev or HSIL | 12 | 12 | 100.0 | 20 | 20 | 100.0 |
| TOTAL | 109/244 (44.7%) | | | | | |

Table 6.5 Comparison between the HR-HPV results using HCAII and the ThinPrep® and conventional Pap smear results for the 246 women in the *study set*.

NT = not tested; n= number tested

Women under 30 years were more likely than older women to test positive for HR-HPV using HCAII. The HR-HPV positivity rate in women <30 years was 62.3% (68/109) and this gradually declined with age to 20% (13/65) in women over the age of 40 (Table 6.6). Women under 30 were also more likely to have high-grade cytological abnormalities. Two women aged 23 years had histologically confirmed CIN 3.

| Age (years) | Number of samples | HPV Pos by HCAII | % HPV Pos | Cytology | U/S or INAD | Neg or WNL | B/L or ASCUS | Mild or LSIL | Mod | Sev |
|----------------|-------------------------|------------------------|-------------------------|----------|----------------|---------------|-----------------|--------------------|---------|-----|
| | | | | | | | | | Or HSIL | |
| <25 | 37 | 28 | 75.7 | CP | 2 | 21 | 6 | 6 | 1 | 1 |
| | | | | TP | 0 | 22 | 2 | 10 | 3 | |
| 25–29 | 72 | 40 | 55.5 | CP | 3 | 45 | 9 | 11 | 2 | 2 |
| | | | | TP | 2 | 44 | 7 | 13 | 6 | |
| 30–39 | 71 | 28 (1NT) | 40 | CP | 6 | 46 | 9 | 6 | 1 | 3 |
| | | | | TP | 1 | 55 | 4 | 2 | 9 | |
| 40–49 | 42 | 10 | 23.8 | CP | 2 | 30 | 6 | 3 | 0 | 1 |
| | | | | TP | 1 | 32 | 4 | 4 | 1 | |
| 50–59 | 21 | 3 (1NT) | 15 | CP | 1 | 15 | 4 | 0 | 0 | 1 |
| | | | | TP | 1 | 18 | 0 | 1 | 1 | |
| >60 | 3 | 0 | 0 | CP | 0 | 2 | 1 | 0 | 0 | 0 |
| | | | | TP | 0 | 2 | 1 | 0 | 0 | |
| TOTAL | 246 | 109 | 44.7 | | | | | | | |

Table 6.6 Age breakdown of the 246 women in the *study set* with corresponding cytology results and HPV positivity by HCAII

CP = conventional Pap

TP = ThinPrep®

NT = not tested

6.3.5 Detection of HPV DNA in cervical samples from women with no previous cytological abnormalities

HR-HPV DNA was detected by HCAII in 17.3% of smears obtained from the 220 age-matched women in the *control set* (i.e those with no previous cytological abnormality). Approximately 16% of women with normal cytology (by either Pap smear or TP) were found to harbour HR-HPV types in the cervix (Table 6.7). The results also suggested that HPV prevalence was higher in women with negative cytology that had a previous abnormality (30–34% as shown in Table 6.5) compared to those that had never had any cytological abnormality when either Pap smears ($\chi^2 = 15.3546$ on 1 degree of freedom, $P < 0.05$) or TP ($\chi^2 = 12.4175$ on 1 degree of freedom, $P < 0.05$) were assessed.

| | CONTROL SET (n=220) | | | |
|-----------------|------------------------|------|-----------------|------|
| | Conventional Pap smear | | ThinPrep® smear | |
| | HPV Pos | % | HPV Pos | % |
| U/S or INAD | 0/1 | | 0/3 | |
| Neg | 38/219 | 17.4 | 33/209 | 15.8 |
| B/L or ASCUS | 0 | | 0/3 | |
| Mild or LSIL | 0 | | 4/4 | |
| Mod/Sev or HSIL | 0 | | 1/1 | |
| TOTAL | 38/220 (17.3%) | | | |

Table 6.7 Comparison between the HR-HPV test results using HCAII and the ThinPrep® and conventional Pap smear results for the 220 age-matched women in the *control set*.

6.3.6 Follow-up results for the Study Set

The recommended recall interval for 5 women of the *study set* lies outwith the study period and no follow-up results are available. These women will however continue to be followed up. In addition, 10 women have defaulted to date from follow-up despite at least one reminder, but will still be subject to failsafe follow-up.

Of the 231 remaining women, the first repeat Pap smear results showed 158 women with normal cytology, 35 with borderline nuclear changes, 26 women with mild dyskaryosis, 4 with moderate dyskaryosis and 8 with severe dyskaryosis.

6.3.6.1 Women with a negative cytology result on the first repeat smear

Of the 158 women who had a normal smear (WNL) following their index smear, 55 were HR-HPV positive (35%) and 101 were HR-HPV negative (Table 6.8). Two thirds (66/101) of the HPV negative women had a further negative smear. Although no further follow-up was available for 25 of the 36 remaining HPV negative women, only three women with satisfactory smears had subsequent borderline cytology.

Twenty-two of the 55 (40%) HR-HPV positive cases had another negative smear. Further follow-up in six of these HPV positive women has shown three women to have subsequent borderline smear reports and three women to have histologically confirmed CIN 2 or CIN 3.

Three women were referred immediately to colposcopy on clinical grounds after the result of their index smear, including one HR-HPV positive women less than 30 years old. Histological assessment of tissue removed from the cervixes of these women did not reveal any neoplastic changes.

Fifteen out of 158 women had an inadequate result in the second repeat smear. Eight out of nine women with subsequent investigations had negative Pap cytology. Two of these women have had further follow-up: one HR-HPV negative woman, >30 years, had a subsequent negative smear and one HR-HPV positive woman over 30 years old had an inflammatory biopsy. The other woman was HR-HPV positive, more than 30 years of age and had subsequent B/L cytology followed by an inadequate smear report.

| Index smear | 1 st repeat smear | 2 nd repeat smear | Age | HR-HPV by HCAII | |
|---------------------|------------------------------|------------------------------|--------------|-----------------|------------|
| | | | | Pos | Neg |
| B/L or mild (n=246) | Neg (n=158) 2NT | Colposcopy (n=3) | <=30 | 1 | 0 |
| | | | >30 | 1 | 1 |
| | | | TOTAL | 2 | 1 |
| | | None (n=42) 1NT | <=30 | 15 | 8 |
| | | | >30 | 2 | 16 |
| | | | TOTAL | 17 | 24 |
| | | U/S (n=15) | <=30 | 3 | 4 |
| | | | >30 | 5 | 3 |
| | | | TOTAL | 8 | 7 |
| | | Neg (n=89) 1NT | <=30 | 11 | 20 |
| | | | >30 | 11 | 46 |
| | | | TOTAL | 22 | 66 |
| | | B/L (n=7) | <=30 | 1 | 2 |
| | | | >30 | 3 | 1 |
| | | | TOTAL | 4 | 3 |
| | | Mild (n=2) | <=30 | 2 | 0 |
| | | | >30 | 0 | 0 |
| | | | TOTAL | 2 | 0 |
| TOTAL | | 158 (2NT) | | 55 | 101 |

Table 6.8 Follow-up cytology in relation to HPV status in 158 women with a negative result on their first repeat smear

NT= not tested

89 women had two consecutive negative smears after the index smear and 66 (74.2%) of these were HR-HPV negative. Six out of 89 women have had further negative follow-up.

Seven women had a borderline result in the second repeat smear. Four were HR-HPV positive and three were HR-HPV negative. One HPV-positive woman was found to have CIN3 on biopsy at follow-up. Follow-up of the others, over 1–10 months failed to detect cervical neoplasia.

Two women, under 30 years of age, had a mildly dyskaryotic smear result on the second repeat smear. Both were HR-HPV positive, have had subsequent negative smears and are due further follow-up.

No moderately or severely dyskaryotic smears were found on the second repeat smear in this group.

6.3.6.2 Women with a borderline cytology result on the first repeat smear

Of the 35 women who had a borderline smear following their index smear, 17 (48.6%) were HR-HPV positive. Thirteen out of 17 (76.5%) were less than 30 years old (Table 6.9). Two women were referred immediately to colposcopy on clinical grounds after the B/L result of their index smear: a 26 year old, HR-HPV positive woman was found to have histologically confirmed CIN 2.

Five out of 35 women with further cytological follow up had a second repeat smear reported as inadequate. All four HR-HPV negative women had subsequent negative smears. The HR-HPV positive woman is awaiting further follow up.

Sixteen out of 35 women had a negative second repeat smear. Three out of six HR-HPV positive women under 30 years old had subsequent smears, including three with negative smears and one woman with a mildly dyskaryotic smear, confirmed as CIN 1–2 on biopsy. Eight out of 9 HR-HPV negative cases were women 30 years of age or more. Three have had subsequent negative follow up smears.

Three out of seven women with borderline changes on the second repeat smear were HR-HPV positive. Two of these women were less than 30 years old and had histologically confirmed CIN 1 or CIN 3. Two out of four HR-HPV negative women over 30 years of age had biopsies within normal limits. The other two HR-HPV negative women were found to have CIN 1 lesions. The presence of low-grade HPV types was investigated in LBC specimens collected from both women with HR-HPV negative CIN 1 using conventional GP5+/6+ PCR. DNA from low-risk genotypes was not detected by this method in extracted DNA from either of the specimens.

| Index smear | 1 st repeat smear | 2 nd repeat smear | Age | HR-HPV by HCAII | | Histology |
|---------------------|------------------------------|------------------------------|-------|-----------------|-----|---|
| | | | | Pos | Neg | |
| B/L or Mild (n=246) | B/L (n=35) | U/S (n=5) | <=30 | 1 | 2 | |
| | | | >30 | 0 | 2 | |
| | | | TOTAL | 1 | 4 | |
| | | Neg (n=16) | <=30 | 6 | 1 | |
| | | | >30 | 1 | 8 | |
| | | | TOTAL | 7 | 9 | |
| | | B/L (n=7) | <=30 | 2 | 0 | CIN1 x1 CIN 3 x1 Inflam x1 CIN 1 x2 WNL x2 |
| | | | >30 | 1 | 4 | |
| | | | TOTAL | 3 | 4 | |
| | | Mild (n=3) | <=30 | 1 | 0 | Neg x1 CIN1 x2 |
| | | | >30 | 2 | 0 | |
| | | | TOTAL | 3 | 0 | |
| | | Mod (n=1) | <30 | 1 | 0 | CIN1 x1 |
| | | | >30 | 0 | 0 | |
| | | | TOTAL | 1 | 0 | |
| | | Sev (n=1) | <=30 | 1 | 0 | CIN3 x1 |
| | | | >30 | 0 | 0 | |
| | | | TOTAL | 1 | 0 | |
| | | Colposcopy (n=2) | <=30 | 1 | 0 | CIN2 x1 Inflam x1 |
| | | | >30 | 0 | 1 | |
| | | | TOTAL | 1 | 1 | |
| TOTAL | | 35 | | 17 | 18 | |

Table 6.9 Follow-up cytology in relation to HPV status for 35 women with a borderline cytology result on their first repeat smear

Three women had mild results on the second repeat smear. All were HPV positive. Both women over 30 years had CIN 1 on biopsy and the younger women had a subsequent negative smear.

One HR-HPV positive women under 30 years of age had a moderately dyskaryotic second repeat smear. This was confirmed as CIN 1 by biopsy. Another HR-HPV positive women under 30 years of age had a severely dyskaryotic smear on the second repeat. This was histologically confirmed as CIN 3.

6.3.6.3 Women with mild dyskaryosis on the first repeat smear

Of the 26 women who had a mildly dyskaryotic smear after their index smear, 22 (84.6%) were HR-HPV positive. 65.4% (17/26) were less than 30 years old (Table 6.10). On clinical grounds, twelve were immediately referred to colposcopy. 10 of these 12 women were HR-HPV positive and were found to harbour neoplastic lesions (CIN 3 three times, CIN 2 twice and CIN 1 five times). Histological examination of biopsy material from both HR-HPV negative cases showed inflammatory changes only (one biopsy has been independently reviewed).

Five out of 26 women had second repeat smears that were reported as negative. Two of three HR-HPV positive women were less than 30 years old: one woman had a subsequent B/L smear which was confirmed as CIN 1 by histological assessment and the other HR-HPV positive woman had a subsequent negative smear. One woman over 30 years of age was HR-HPV negative and had a subsequent negative smear followed by a B/L smear report.

Seven women had subsequent borderline or mildly dyskaryotic smears on the second repeat. All were HCA positive. Six of these women were referred for colposcopic examination and the biopsy results showed WNL twice, CIN 1 twice, CIN 2 once and CIN 3 once. The other HR-HPV positive woman was over 30 years of age and had two subsequent negative smears.

Two HR-HPV positive women had moderately dyskaryotic smears. One had CIN 1 on biopsy and the other woman had two subsequent negative smear reports. There were no inadequate or severe reports in the second repeat smears.

| Index smear | 1 st repeat smear | 2 nd repeat smear | | HR-HPV by HCAII | | Histology |
|---------------------|------------------------------|------------------------------|-------|-----------------|-----|--------------------------------------|
| | | | Age | Pos | Neg | |
| B/L or Mild (n=246) | Mild (n=26) | Neg (n=5) | <=30 | 2 | 1 | CIN1 x1 |
| | | | >30 | 1 | 1 | |
| | | | TOTAL | 3 | 2 | |
| | | B/L (n=3) | <=30 | 3 | 0 | WNL x2 CIN1/BL x1 |
| | | | >30 | 0 | 0 | |
| | | | TOTAL | 3 | 0 | |
| | | Mild (n=4) | <=30 | 2 | 0 | CIN2 x1 CIN3 x1 CIN1 x1 |
| | | | >30 | 2 | 0 | |
| | | | TOTAL | 4 | 0 | |
| | | Mod (n=2) | <=30 | 1 | 0 | CIN1 x1 |
| | | | >30 | 1 | 0 | |
| | | | TOTAL | 2 | 0 | |
| | | Colposcopy (n=12) | <=30 | 9 | 1 | CIN1 x4 CIN2 x2 CIN3 x3 Inflam x1 |
| | | | >30 | 1 | 1 | |
| | | | TOTAL | 10 | 2 | |
| TOTAL | | 26 | | 22 | 4 | |

Table 6.10 Follow-up cytology in relation to HPV status in 26 women with mild dyskaryosis on their first repeat smear

6.3.6.4 Women with moderate or severe dyskaryosis on the first repeat smear

Twelve HR-HPV positive women had moderate or severe dyskaryosis on their first repeat smear (Table 6.11). All were HR-HPV positive. Six were less than 30 years old. Three out of 4 HR-HPV positive women with moderate dyskaryosis on the first repeat smear were less than 30 years old and had CIN 2 once and CIN 3 twice on biopsy. The other HR-HPV positive woman was older than 30 and had CIN 2 on biopsy.

Three out of eight HR-HPV positive women with severe dyskaryosis on the first repeat smear were less than 30 years of age. All had CIN 3 confirmed by histological examination. The other five HR-HPV positive women were over 30 years old. One woman had CIN 1; one had CIN 2 ; three women had CIN 3 and one had squamous cell carcinoma (SCC). In summary, the colposcopic biopsies from all 12 women showed CIN 1, CIN 2 three times and CIN 3 seven times and one case of SCC.

| Index smear | 1 st repeat smear | 2 nd repeat smear | Age | HR-HPV by HCAII | | Histology |
|---------------------|------------------------------|------------------------------|-------|-----------------|-----|---------------------------------|
| | | | | Pos | Neg | |
| B/L or Mild (n=246) | Mod (n=4) | | <=30 | 3 | 0 | CIN 2 x1 CIN 3 x2 |
| | | | >30 | 1 | 0 | CIN2 x1 |
| | | | TOTAL | 4 | 0 | |
| | Sev (n=8) | | <=30 | 3 | 0 | CIN3 x3 |
| | | | >30 | 5 | 0 | CIN1 x1 CIN2 x1 CIN 3 x2 SCC x1 |
| | | | TOTAL | 8 | 0 | |
| TOTAL | | 12 | 12 | 0 | | |

Table 6.11 Follow-up results in relation to HPV status in 12 women with moderate or severe dyskaryosis on their first repeat smear

In summary, 64.6% of women who had a previous B/L smear had a negative smear on their return visit and two-thirds of these were HR-HPV negative. A further 64.7% of these HR-HPV negative women remained cytologically normal on their second return visit. In the remaining third, three of 11 women with satisfactory smears had a further B/L smear report. 14.2% of women who had a previous B/L smear continued to show B/L nuclear changes on the first early repeat smear. Of the HR-HPV negative women in this group, two had a biopsy showing CIN1. 10.6% of women had a mildly dyskaryotic smear on first early repeat, and 84.6% of women in this group harboured HR-HPV DNA in their cervixes. All HR-HPV negative cases were found to have no cytological abnormality on biopsy or subsequent smear. 4.9% of women had a moderately or severely dyskaryotic smear on first early repeat which was confirmed on biopsy. All cases of moderate or severe dyskaryosis were HR-HPV positive.

6.4 Discussion

6.4.1 Liquid-based cytology

For accurate diagnosis of cervical disease, a truly representative cervical sample is needed. Since TP was approved by the United States Food and Drug Administration (FDA) in 1996, as a replacement for conventional Pap smears, several studies have evaluated the effectiveness of this method to detect significant cervical disease. Early comparative studies between TP and Pap smears found TP to yield fewer diagnostic cells (Bur *et al.*, 1995) and no increase in the detection of cervical abnormalities was reported (Aponte-Cipriani *et al.*, 1995). Indeed, in the first population-based comparison study by Hutchinson *et al.*, (1999) considerably more TP slides were diagnosed as ASCUS (equivalent to B/L changes) compared to Pap smears. However, that particular study involved use of the β -model of the ThinPrep® processor that allows for less specimen variability and does not place as many cells on each slide. In contrast, more recent studies using improved TP technology, such as the FDA-approved TP2000 slide processor, have shown good correlation between TP and Pap smear results. In the study reported here, using TP2000, 87.5% agreement was found between satisfactory TP and Pap smear results. This is similar to other studies where the level of agreement has ranged from 85.4% to 94.6% (Ferenczy *et al.*, 1996, Roberts *et al.*, 1997; Wilbur *et al.*, 1994; Bur *et al.*, 1995). The use of the TP test in both split-sample and direct-to-vial studies has resulted in significantly increased detection of low-grade and high-grade squamous intraepithelial lesions (Roberts *et al.*, 1997; Wilbur *et al.*, 1994; Guidos and Selvaggi, 1999; Yeoh *et al.*, 1999) and an increase in biopsy-proven high-grade disease (Papillo *et al.*, 1998). In the split-sample study reported here, analysis of 800 TP and Pap smear preparations showed that TP allowed for a more accurate assessment of cervical cytology with the detection of eight more cases of low-grade abnormalities and 13 additional cases of high-grade dyskaryosis. In 56.7% (51/90) of cases where diagnostic agreement was not found between both smear preparations, this was attributed to the TP method detecting a more severe abnormality than the Pap smear. This included 3 cases that appeared normal by Pap smear assessment but high-grade abnormalities were

detectable by TP. Indeed, the conventional Pap smear has been shown to have a false negative rate of 15% for the detection of cervical abnormalities whereas cytological assessment by TP has a false negative rate of only 3.9% (Wilbur *et al.*, 1994). Weintraub and Morabia (2000) concluded that there is a 2.2 times greater chance of having a positive diagnosis with TP compared to conventional Pap smears and a 1.86 times greater chance of detecting HSIL using this screening method.

During TP slide preparation, the LBC sample is concentrated with obscuring artefacts being removed from the specimen, whilst preserving cell morphology, before the cells are displayed as a monolayer, evenly distributed within a 20mm diameter circle on the slide. Thus, many of the problems associated with unsatisfactory conventional Pap smear reports are eliminated thus producing better cell preparations for morphological analysis. In the study reported here, TP resulted in improved diagnostic quality as shown by the reduction in unsatisfactory smear reports compared to the Pap smear (7.3% to 2%). This result concurs with the findings of other larger split-sample studies where TP reduced the number of inadequate smear reports by as much as 94% (Roberts *et al.*, 1997; Aponte-Cipriani *et al.*, 1995). Such reduction in the number of inadequate smear reports clearly has benefits for both patients and clinicians at the primary care level by reducing the number of repeat patient visits required during follow-up. In the study reported here, TP also caused a reduction in the number of B/L smear diagnoses compared to the conventional Pap smear (7.9% to 4.2%). As a result of improved TP sample preparation, the majority of samples that were diagnosed as B/L by Pap screening had been classified as either negative (n=32) or LSIL (n=10) by TP. In addition, two cases that had been diagnosed as B/L by Pap smear showed high-grade abnormalities on TP analysis. Although the number of B/L smears was reduced by approximately half by TP analysis, more were HPV positive (48.3% to 54.5%). Although this increase in HPV positivity is not statistically significant ($\chi^2 = 0.3307$ on 1 degree of freedom, $P = 0.5652$) it may suggest that B/L abnormalities detected by TP that are also HPV positive are more likely to be clinically significant.

Unfortunately TP slide preparations are more expensive than Pap smears. Both the reagents and hardware are expensive and the consumables are non-recyclable. Additionally, other manipulations might be required in the laboratory to get a satisfactory smear, which further increases the cost. In a six-month study by Yeoh and colleagues in 1999, 82% of practices that were given an informed choice between ThinPrep® and Pap, chose to convert to ThinPrep® smear preparations. The main reason stated by the other clinics for not converting was cost. Other problems might include the shelf life and storage of supplies in clinics and both the practicalities and cost of transporting alcohol-containing specimens from the clinic to the laboratory. Additionally, considerable retraining of laboratory staff is required before they can be competent at screening TP monolayer smears and education of smear-takers is necessary to inform them of the method required for TP cervical specimen collection. Nevertheless, due to the reduction in both false negative and false positive results, long-term savings are possible through more effective use of medical resources. A decrease in the number of inadequate smears and more efficient management of women with low-grade abnormalities would have additional cost savings. If the study reported here had been performed in clinical practice, where cervical specimens had been direct-to-vial instead of being split samples collected after conventional Pap smear preparation, it is likely that the inadequate TP smear rate in this study would have been even lower.

In June 2000, the National Institute for Clinical Excellence (NICE) report (www.nice.org) concluded that there was insufficient evidence to justify the national implementation of LBC for cervical screening. However, a six-month multi-centre LBC demonstration project has recently been completed in Scotland and a 12-month multi-centre trial is currently underway in England, to assess the effects of introducing such technology into the cervical screening programme.

6.4.2 HPV testing

Several small clinical studies showed that HR-HPV DNA positivity using the first generation HCAI, improved the detection of high-grade cervical neoplasia in women with abnormal cytology compared to Pap cytology alone (Hatch *et al.*, 1995; Cox *et*

al., 1995; Farthing *et al.*, 1994). PreservCyt® samples were also shown to be suitable for HPV testing using HCAI, with increased HPV DNA detection in LBC samples compared cervical specimens collected into Digene STM at the same time (45% and 36.5% respectively). In more recent studies, the more sensitive second generation test (HCAII) has been shown to have 70% sensitivity for the detection of all grades of CIN and more than 90% sensitivity for detecting CIN 2 or above (Mould *et al.*, 2000). In the study reported here, HCAII detected DNA from HR-HPV genotypes in 25.8% of all the LBC samples tested. All samples associated with moderate or severe dyskaryosis on TP analysis were HR-HPV positive and >80% of samples associated with mildly abnormal smears were found to harbour HR-HPV DNA. Using HCAII, HR-HPV prevalence in the study set (i.e. women with a previous B/L smear abnormality) was 44.3% and 17.3% in the age-matched control set (i.e. women with no previous history of cervical smear abnormality).

Two large studies in the United States have provided evidence that HPV testing is suitable for triaging women with minimal smear abnormalities who have underlying CIN lesions. Firstly, results from 3488 women (mean age = 29 years) with ASCUS cytology enrolled in the ALTS trial, showed that HPV testing had 96.3% sensitivity for the detection of CIN 3 or above but 56.1% of the women would be referred to colposcopy (Solomon *et al.*, 2001). However, the predictive value of a negative test for detection of CIN 2 was shown to be 98.9%. Secondly, a study involving 46,009 women (mean age = 38 years) attending routine Pap screening at the American health care organisation, Kaiser Permanente, found that 38.8% of women with ASCUS showed high-grade cervical neoplasia on histological examination and 68.6% of women with either ASCUS or LSIL harboured high-grade cervical neoplasia (Kinney *et al.*, 1998). HPV testing by HCAII was performed on LBC specimens collected from 995 women with ASCUS and was concluded to be 89.2% sensitive for identifying histologically-confirmed high-grade CIN (Manos *et al.*, 1999). It was concluded that triage based on HPV testing would reduce the number of colposcopy referrals and follow-up appointments required compared to the current practices. Similar results have been found by Fait *et al.*, (2000), where colposcopy examination of 503 women with ASCUS or LSIL diagnosed on two

consecutive Pap smears would have been reduced to 24.6% of cases if women harbouring HR-HPV DNA only had been referred while still maintaining sensitivity of 87% for the detection of CIN 2 and CIN 3.

While there is evidence to suggest that HPV testing is useful in triaging women with B/L smear results, several workers have concluded that there was limited potential for HCAII to direct decisions about the clinical management of women with low-grade squamous intraepithelial lesions (LSIL, approximates to mild dyskaryosis). The ALTS Group (2000) found 82.9% of women with LSIL diagnosis from Pap smears harboured HPV DNA by HCAII testing and Rebello *et al.*, (2001) found the overall test sensitivity and specificity of HCAII to be 94% and 39% respectively for detecting underlying high-grade CIN in women with mildly abnormal smears. A repeat smear at six months has shown better sensitivity and specificity for the detection of underlying high-grade CIN than HPV testing by PCR in women with mild dyskaryosis (Cruickshank *et al.*, 1999). In the study reported here, most of the woman enrolled had previous B/L smears, not mild dyskaryosis, and the results suggest that HCAII testing from LBC samples collected from such women could be used to predict the absence of progressive disease.

Few studies investigating HPV testing in triage of women with B/L or mild cytological abnormalities have provided information on long-term follow-up and the incidence of high-grade CIN. Follow-up data from women enrolled in the *study set* suggests that the predictive value of a negative HPV result (i.e. the likelihood that a woman without HPV infection does not have high-grade CIN) is very high. 65.7% of women with a previous B/L abnormality had subsequent negative cytological follow up and 66.3% of these were HR-HPV negative. Two thirds of these HR-HPV negative women remained cytologically normal on their second repeat visit. If all HR-HPV negative women had been returned to routine recall only two cases of CIN1 would have been missed but 160 repeat visits would have been saved. In a similar sized study by Ziehlinski *et al.*, (2001a), the overall sensitivity of a HR-HPV positive test for a CIN 2/3 lesion at the first colposcopy visit was 96.3%; the specificity was 60.2% and the PPV was 20.6%. However, the high NPV (99.3%) of a

HPV test reinforced its potential use as a management strategy for women with B/L or mild dyskaryosis. Although the PPV was shown to be higher in women younger than 40, the NPV was found not to be affected by age variation. In another recent study of Dutch women with mild to moderate or severe dyskaryosis, Nobbenhuis and colleagues (1999) showed that those who did not harbour HR-HPV never developed CIN 3 and only women with persistent HR-HPV infection showed clinically progressive cervical disease. A repeat Pap smear after 6 months has been shown to be less significant than a repeat HPV positive test for identifying women at risk of developing CIN 3 and suggestions have been made that that only women with a second HPV positive result should be sent for colposcopy whereas the screening interval could be considerably increased for HR-HPV negative women (Nobbenhuis *et al.*, 1999).

A practical triage strategy is required for women who have minimally abnormal smears. Possible approaches include repeat cytology and/or HPV testing or referral for colposcopy and biopsy. Based on the results from this investigation a possible triage algorithm is shown in Figure 6.3. This proposal is based on reflex HPV testing using HCAII from residual LBC fluid for all women who have a B/L smear. Since the median duration of HR-HPV clearance in women with B/L or mild dyskaryosis has been shown to be longer than 6 months (Ho *et al.*, 1998; Nobbenhuis *et al.*, 1999; Ziehlinski *et al.*, 2001a) and persistent infection is necessary for the development of cervical neoplasia, a repeat HPV test after six months would be considered redundant. A repeat smear and HPV test after 12 months would provide more accurate information regarding the risk of disease progression. At that point, women with a second HR-HPV positive result in association with the repeat LBC smear would be recommended for colposcopic examination. HR-HPV negative women with abnormal cytology would also be recommended for immediate colposcopy referral to minimise the risk of a false negative HPV test result. However, HR-HPV negative women with no evidence of any cytological abnormality would be safely returned to routine screening. This approach would reduce the number of colposcopy referrals and the number of repeat smears. By inclusion of a HPV test, over treatment of women with B/L smears would be limited. If, in addition, the screening interval

was increased for women who have been HR-HPV negative on both occasions, a marked decrease in the current expenditure associated with follow-up would result. It has also been suggested that fewer women would be lost to follow-up if HPV DNA testing was used rather than repeat smear testing (Lytwyn *et al.*, 2000).

Review of the literature has shown that there have been some disparate results reported in studies on this topic. Possible reasons for this is that it is difficult to compare studies in the USA to the UK because cervical screening populations and procedures are different and there are important differences between the classification of cytological abnormalities. Additionally, differences in the HPV testing methodology and the age distribution of the women studied may also vary between different studies. In the study reported here, the HPV prevalence was highest in women under 30 years old (62.3%) and declined with increasing age to just over 11% in women over 40 years of age. In view of similar findings by others, it has been suggested that HPV testing would be cost-effective in women with low-grade cervical abnormalities only if women over 30 years of age who are less likely to have transient HPV infection were tested (Cuzick, 1998). However, in this reported investigation, nine of 20 cases showing high-grade abnormalities were detected in TP monolayers from women under 30. Seven of these 9 women were referred to colposcopy on the basis of mild, moderate or severe dyskaryosis detected by the conventional Pap smear. However, TP analysis was more effective than the Pap smear for identification of significant cervical disease in the other two women: one woman with mild dyskaryosis by Pap smear analysis had two subsequent smears (reported as WNL and B/L) before CIN 1 was identified by histological examination. The other woman aged 25 years was found to be WNL by Pap cytology and has not had further follow up to date. The number of women over 60 years enrolled into this study was small and therefore it is not possible to confirm or dispute previous observations of increased detection of cytological abnormalities in post-menopausal women (Herrero *et al.*, 2000).

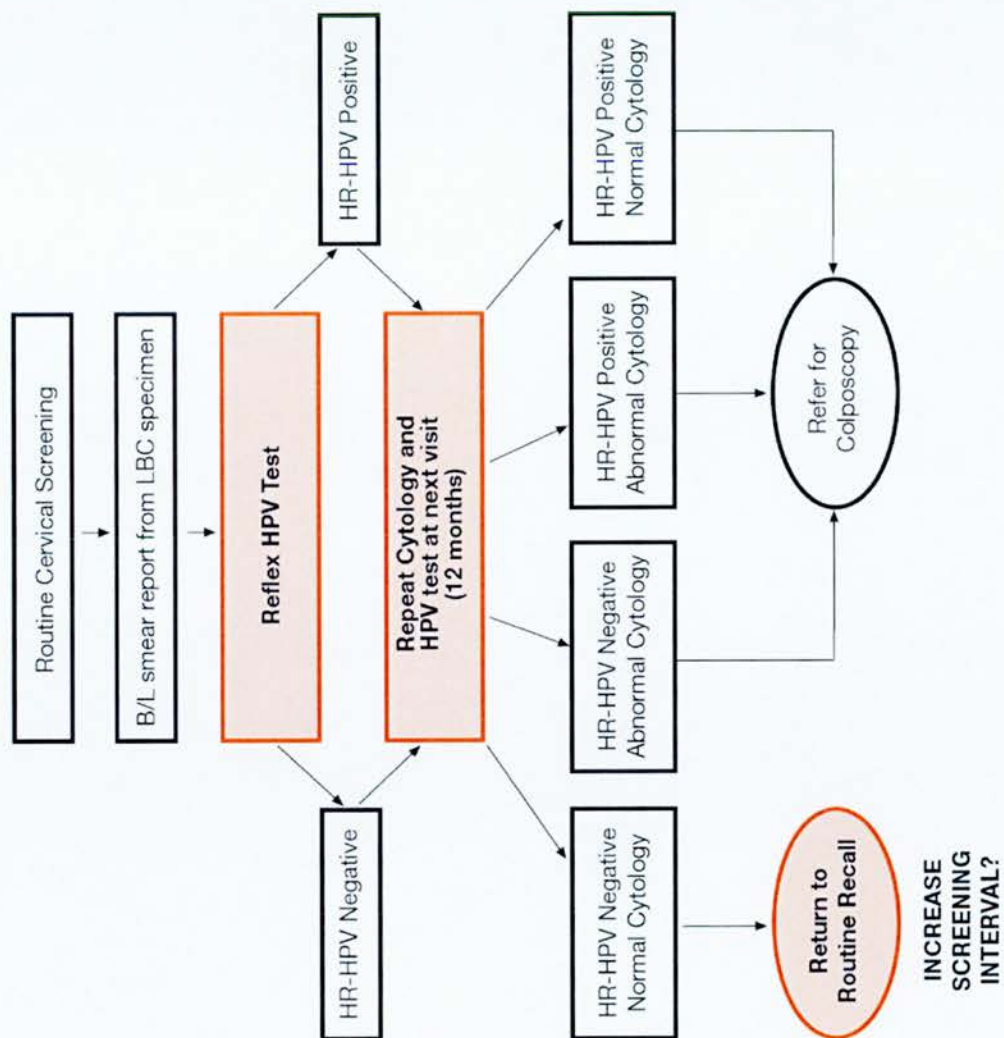


Figure 6.3 Proposed triage algorithm for women with borderline smear reports

Finally, the HTA systematic review (Cuzick *et al.*, 1999a), recommended that HPV testing be introduced on a pilot basis for women with B/L and mildly dyskaryotic smears and a large trial of HPV testing should be undertaken in conjunction with other new technologies (such as LBC and computer-assisted cytology reading) to determine the best way to integrate them into ongoing screening programmes (Cuzick *et al.*, 2000). Many of these issues will be addressed by the pilot studies of HPV testing, in conjunction with LBC, for women with B/L or mildly dyskaryotic smears which began in England in March 2001 (Dr. E. McGoogan and Dr. H.A. Cubie, personal communication).

6.5 Conclusion

When this study began, the Scottish Office had not given approval for LBC samples to be used as diagnostic specimens, so the TP specimens collected could not be used for cytology diagnosis. Despite the potential bias introduced by preparing the Pap smear first, improved specimen adequacy provided in part by the sampling device; removal of artefacts; specimen mixing and the random selection of cells during TP monolayer preparation allowed better detection of clinically important cervical disease and a reduction in the number of unsatisfactory and B/L smear diagnoses. From a laboratory perspective, improved specimen adequacy and quality provided by the ThinPrep® test significantly reduced the time required for screening and the problems associated with interpretation (Dr. E. McGoogan, personal communication). With release of the fully automated TP3000 system which can process four batches of 80 slides per 8-hour shift the problem of single specimen handling has been addressed. In clinical practice, where the entire cell sample would be transferred into the PreservCyt® fluid, the performance of TP would be expected to be even more impressive. Although the initial expenditure to implement LBC may be high, in the long term, there are cost savings to be made through reduced cytology recalls and colposcopy referrals. More importantly, if improved detection of cervical disease has a reducing effect on the national cervical cancer incidence rate, then more costly tests become affordable due to the savings made from cancer management. Data generated from the Scottish LBC demonstration projects will provide further important information about the implications of introducing liquid-based cytology technology into the NHS cervical screening programme.

The introduction of LBC allows the cervical material collected to be used for additional tests from a single specimen without the requirement for a second patient visit. The results reported here suggest that HPV testing using HCAII may be useful for triage of women with borderline abnormalities by streamlining patient management according to high-risk HPV status. For this purpose, 'reflex HPV testing' may be employed where the LBC specimen is only selected for HPV testing

if a B/L smear result is obtained. Since the predictive value of a HCAII–negative test has been shown to be very high, women with borderline smear diagnoses who do not harbour HR–HPV could be safely returned to routine recall whereas HR–HPV positive women would be referred for further follow–up. A proposed triage algorithm, based on the results from this study, recommends colposcopy referral only when persistent HPV infection is present, as shown by two successive HR–HPV positive results. Single time point HPV test results should be interpreted with caution since many women will be transiently infected, particularly those under 30 years of age. In this context, it is predicted that LBC combined with HR– HPV testing by HCA II would be a cost–effective measure although the results generated from this study’s control set suggest that HPV testing by HCAII is unlikely to be cost–effective in the routine screening of normal women.

From recent reports (Ferenczy and Franco, 2001; Meijer and Walboomers, 2000) the future of cervical screening is likely to include automated cytology and molecular virology methods for the detection of cervical cancer and its precursors and accurate prediction of disease outcome. The technologies described in this chapter hold great promise for achieving this, and ultimately a reduced worldwide cervical cancer incidence rate.

CHAPTER 7

DEVELOPMENT OF AN AUTOMATED PROCEDURE FOR DNA EXTRACTION FROM LIQUID-BASED CYTOLOGY SPECIMENS AND A REAL-TIME PCR SYSTEM FOR HPV DETECTION

7.1 Introduction

The Pap smear has remained relatively unchanged since it was introduced 50 years ago. It is expensive, labour intensive, time consuming, requires highly skilled staff and has many inherent opportunities for failure. The advent of LBC to replace conventional Pap smears, will address many of the sensitivity issues due to sampling and preparation errors associated with conventional smears. Testing for HR-HPV in conjunction with LBC may further improve the cervical screening programme by identification of women at increased risk of cervical disease progression or for triage of B/L or low-grade smear abnormalities. This, however, depends on a rapid, sensitive test for the detection of HR-HPV genotypes. So far, the best methodology for this purpose has not been determined. Neither current consensus PCR methods nor the second generation HCAII test can distinguish between individual HPV genotypes or detect infection with more than one type without performing additional procedures. The ability to distinguish between different HR-HPV types in a single assay and quantify the amount of DNA present would be advantageous for laboratory testing. Thus, to distinguish HR-HPV types present in clinical samples, other molecular technologies including automated DNA extraction protocols and rapid HPV detection methods are required. As the clinical use of LBC becomes more widespread for the collection and cytological assessment of cervical specimens, the ability to test the residual cells from large numbers of LBC specimens for HR-HPV types becomes possible.

For molecular tests to be applicable in a routine diagnostic laboratory, sample preparation and DNA extraction should be as simple as possible. The use of robotics enhances rapidity and automated nucleic acid extraction systems are now commercially available, such as the BioRobot® 9604 (QIAGEN Ltd, Crawley, East

Sussex), MagNA Pure LightCycler (Roche Diagnostics Ltd., Lewes, East Sussex) and the RoboAmp® (MWG Biotech, Milton Keynes). The advantages of such commercial systems include the potential for high throughput of clinical samples for DNA extraction with reliable results and consistent yields while reducing the 'hands-on' time involved.

The BioRobot® 9604 is a workstation for the extraction of nucleic acid from clinical specimens prior to PCR using QIAamp® silica-based membrane technology in a 96-well plate format. Currently, licensed BioRobot® 9604 protocols are available for automated DNA extraction from blood, body fluids, cultured cells and buccal swabs.

The MagNA Pure system uses magnetic glass bead technology for extraction of nucleic acids from blood or cultured cells. However, the most important feature of the MagNA Pure LightCycler (LC) system is that it will automatically set up real-time PCR reaction mixes following the automated DNA purification step. In addition, this system will load the PCR reaction mixes into the LightCycler capillaries in the LC carousel. In a recent comparative assessment of these automated extraction systems, both methods were found to be equivalent for the extraction of herpes simplex virus (HSV) DNA from swab specimens prior to LC PCR, since the number of amplification cycles required for HSV detection differed by no more than 1.5 between both methods (Espy *et al.*, 2001).

Conventional PCR sample preparation and final detection using agarose gel electrophoresis does not lend itself to automation, and therefore is not suitable for large specimen numbers. Since kinetic PCR analysis by real-time monitoring of PCR amplicon formation was first described by Higuchi *et al.*, (1992), the potential of PCR testing has been revolutionised due to increased PCR rapidity, reduced contamination risks due to the use of closed PCR reaction vessels and, if required, the ability to perform quantitative PCR. Although several manufacturers are now producing real-time PCR equipment, the ABI PRISM® 7700 Sequence Detection System (Perkin Elmer Applied BioSystems, Warrington, Cheshire) and the LightCycler (now supplied by Roche Diagnostics Ltd.) are the most used.

The LightCycler instrument allows rapid automation of PCR (approximately 30 cycles in 30 minutes) by precise air-controlled temperature cycling and real-time monitoring of amplicon production after each PCR cycle using either SybrGreen I™ or fluorogenic probe technology (Section 3.2). SybrGreen I™ is a fluorescent dye which, during each round of PCR amplification, binds to the minor groove of accumulating dsDNA. To detect the increase in fluorescence that occurs as specific PCR product accumulates, a microlens at the end of each capillary detects the increase in fluorescence as product accumulates and bounces the light from a blue light-emitting diode (LED) into the sample. A photodiode detects the fluorescence signal generated at the end of each primer extension stage. Data from the high precision optics that monitor the amplification reaction can be followed on a computer screen in real-time. Fluorescence melting-curve analysis can be used to identify PCR products with different GC:AT ratios without the need for post-amplification sample manipulations. As the temperature increases, dsDNA becomes denatured and SybrGreen I™ molecules become unbound resulting in a rapid decrease in fluorescence. Alternatively, product detection formats using fluorophore-labelled hybridisation probes can be used for sequence-specific detection of PCR products. In such cases, one primer is designed with an internal label (e.g. Cy5 acceptor). Since real-time PCR uses a much smaller sample volume and combines amplification and detection in closed PCR reaction capillaries, the risk of contamination generated by carry-over of amplified products during post-PCR manipulation is much reduced. Therefore, this methodology has potential compared with other methods for both faster throughput of patient samples and a reduced risk of false positive results.

In contrast, the ABI PRISM® 7700 Sequence Detection System uses TaqMan chemistry for DNA and RNA quantitation (Grove, 1999). In summary, the method uses probes that are end-labelled with two fluorescent dyes (a reporter and quencher). The inherent 5'–3' exonuclease activity of Taq polymerase cleaves the 5'–reporter fluorochrome from the bound probe during the PCR primer extension stage. This event also removes the reporter dye from the proximity of the 3'–quencher dye,

generating a fluorescent signal that is proportional to the amount of product generated in each PCR cycle. A quantitative HPV and β -globin multiplex PCR assay based on this technology was developed by Swan *et al.*, (1997) using a combination of type-specific L1 primers and probes for the detection of HPV 16, 18, 31, 33 and 35. The same group later developed a real-time HPV PCR assay, (using a ABI Prism 7700 Sequence Detection System) for the quantitative detection of HPV16, 18, 6 and 11 (Tucker *et al.*, 2001). A multiplex format was also employed in that study to allow simultaneous amplification of a cellular target to act as an internal PCR control. The multiplex PCR assay performance was tested using extracted DNA from 54 LBC specimens and twelve (22%) were found to be HPV 16 positive.

Since the advent of real-time PCR, the number of publications arising from its application in virology is increasing rapidly. So far, most diagnostic virology investigations have focused on the detection and quantitation of nucleic acid from herpes viruses (Nicoll *et al.*, 2001), including cytomegalovirus (Schaade *et al.*, 2000; Schalasta *et al.*, 2000a; Nitsche *et al.*, 1999), varicella zoster virus (Espy *et al.*, 2000a) and herpes simplex virus (Kessler *et al.*, 2000; Espy *et al.*, 2000b). Due to the sequence relatedness of HSV genotypes, differentiation based on the melting temperature (T_m) of PCR products alone can be difficult (Dr. S. McDonagh, personal communication). However, differentiation between HSV genotypes 1 and 2 (HSV-1 and HSV-2) has been possible using a hybridisation probe designed to target a region of the HSV-2 glycoprotein B gene where there is a G:T mismatch. This yields a lowered probe/product T_m for HSV-1 (69°C) compared to HSV-2 (64°C) allowing clear separation of both genotypes in the same run (Schalasta *et al.*, 2000b).

More specifically, a real-time nested PCR assay has been recently developed for HPV DNA detection using both MY09/11 and GP5+/6+ primers (Strauss *et al.*, 2000). The real-time PCR sensitivity was found to be comparable to that achieved by performing nested PCR on a conventional metal block thermocycler, with 86.1% agreement between both methods. Sequence analysis of PCR positive amplicons

from both real-time and conventional PCR assays showed 70% to contain the same HPV type(s).

7.2 Aims of this study

The aim of this study, was to develop an automated DNA extraction protocol to replace the manual nucleic acid extraction procedure for DNA isolation from routine cervical LBC specimens, so that high specimen throughput could be achieved. A further aim was to apply the LightCycler PCR protocol for HPV detection to DNA extracted from the LBC samples and to test the validity of T_m analysis as a means of differentiating between HPV genotypes. An alternative HPV genotyping assay (RLBA; see Section 2.10) was used to validate the results obtained.

7.3 Methods

7.3.1 Clinical samples

To avoid the need for a conventional smear in addition to the LBC specimen, patient enrolment to this study was delayed until after the Chief Medical Officer's letter relating to LBC in May 1999. Thus, in the first 'direct-to-vial' study in the UK, more than 4700 LBC samples were collected between December 1999 and July 2000 from women attending routine cervical screening clinics in 16 Lothian General Practices. The collection devices were each rinsed in 20mls of PreservCyt® solution and then discarded. Informed consent was obtained for use of the residual samples for detection of microorganisms (Appendix 2) and ethical permission was granted from Lothian Health (LREC No. 1702/98/2/22).

7.3.2 Preparation of ThinPrep® slides

In the UEPD, monolayer smear preparations were made using ThinPrep® 2000 technology, stained conventionally using Pap stain and examined by cytoscreeners trained in the use of LBC and reviewed by trained cytopathologists. Cytological assessment of the monolayers was carried out within the normal turn-around time and the results returned to the GP practices in the usual way.

7.3.3 Processing of LBC specimens for HPV testing

Samples were then sent to the HPV testing laboratory at RCVL and stored at 4°C. To adhere to manufacturers' guidelines, LBC specimens were processed within 21 days receipt at the laboratory. The first 309 samples obtained were processed for both HCAII and LightCycler GP5+/6+ PCR testing following DNA extraction using the manual protocol. For these samples, a 4ml volume was processed for HCAII testing (as outlined in Section 2.7) then the entire residual cell suspension was processed for manual DNA extraction. This was particularly labour intensive and unsuitable for large numbers, so subsequent specimens were processed for DNA extraction only. For all other specimens, the robotic DNA extraction procedure was used. For 873 specimens, the entire residual specimen was processed but a reduced volume of 10ml was processed for 668 samples. Additionally, 50 specimens were processed using

10ml, 5ml and 1ml volumes from the same residual LBC specimen (Table 7.1). Cell pellets were resuspended in 180µl ATL Lysis Buffer (provided by QIAGEN Ltd.) and stored at –70°C until DNA extraction was performed.

| No. of samples | DNA extraction method | HPV detection method |
|----------------|---|----------------------|
| 309 | heat denaturation + NaOH | HCAII |
| | Manual spin columns | Real-time PCR |
| 873 | Robotic extraction of whole sample | Real-time PCR |
| 668 | Robotic extraction of 10mls | Real-time PCR |
| 50 | Robotic extraction of 10, 5, 1ml aliquots | Real-time PCR |
| 1900 | | |

Table 7.1 Summary of the methods used for DNA extraction from 1900 LBC samples and for subsequent HPV DNA detection

7.3.4 Data handling

A study database was constructed containing full cytological, histopathological and clinical histories for all women enrolled and the HPV results obtained. The patients were anonymised and given unique identifiers. The HPV status of the patients did not influence clinical management and the HPV results were not released to the GP practices. To reduce bias, the cytology results were not made available until after the HPV testing had been completed.

7.3.5 DNA Extraction from LBC specimens using the BioRobot® 9604

Automated DNA extraction from LBC specimens involved considerable optimisation of QIAGEN approved protocols in conjunction with the manufacturer and BioRobot Applications Specialists (Section 3.2). Software used for the QIAamp® 96 DNA blood extraction protocol was modified using the conditions used for manual DNA extraction (Section 2.5.2). The main adaptations were the introduction of an initial lysis step at 56°C for one hour (performed by a heat block); robotic addition of proteinase K to samples (that

were contained in a plastic square-well block on the heat block in preparation for lysis) and the introduction of additional mixing steps to ensure sample homogeneity. Samples were eluted twice with Buffer AE to give a final volume of 400µl.

Initial work involved checking for cross-contamination of samples during the extraction procedure by alternating rows or columns of HPV-containing SiHa cells or ATL Buffer across the QIAamp® plate. β-globin PCR was performed on the extracted material to check the efficacy of each extraction run (Section 2.8.2). A maximum of 24 samples was extracted during this validation stage. Due to the small numbers involved, an internal buffer system was applied for delivery of ethanol and wash buffers AW1 and AW2 from internal 'slots' on the BioRobot platform. For clinical specimen extraction, 96 samples were extracted per run and an external buffer system was used with buffers delivered from buffer bottles connected to the system via the main probe.

7.3.6 Real-time GP5+/6+ PCR using the LightCycler

GP5+/6+ PCR was performed on a LightCycler instrument (Idaho Technology Inc, supplied by Biogene Ltd, Kimbolton, Cambridgeshire, UK) using the method outlined in Section 3.3. A HotStart PCR method was used. TaqStart antibody (Sigma-Aldrich Co. Ltd., Dorset, UK) was added directly to the PCR master mix and the mixture was incubated at room temperature for 10 minutes. 1.25µl of DNA template and 3.75µl of PCR master mix was added to each capillary. Sealed capillaries were centrifuged and placed in the LightCycler carousel. Nucleic acid extracted from SiHa cell dilutions (made from stocks containing 1×10^6 cells/ml with 1–2 copies of HPV16 DNA per cell) were included as positive controls and sterile water was included as a negative control. Detailed T_m analysis was performed on PCR positive samples by continuous measurement of the fluorescence as the samples were individually heated at 0.2°C/sec from 50°C to 85°C after a single round of PCR was complete. Samples with a PCR product within the T_m range of the HPV16 or HPV18 controls (mean T_m of 79.1° and 80.9°C (\pm 2SD) respectively) were considered to be positive for these HPV genotypes. For confirmation of the LC PCR results obtained, some samples were tested using the RLBA (Section 2.10).

7.4 Results

7.4.1 Effects of using LBC on cervical cytology results

In this direct-to-vial study, monolayer preparations from LBC samples reduced the inadequate smear rate from around 10% to less than 1% (Table 7.2). In addition, LBC reduced the number of borderline smear diagnoses from 4.1% to 2.3% and increased the detection rate of moderate or severe cytological abnormalities from 1.4% to 2.1%. These results suggest an increase in sensitivity due to the use of LBC smear preparations, although histological assessment would be required to confirm this. Table 7.2 summarises the cytology results for all 4702 LBC specimens collected. The conventional Pap smear results of the 16 GP practices involved in this study during 1998-1999 are also tabulated to allow a comparison of the results obtained. The substantial improvements made by the use of LBC sampling by the reduction of unsatisfactory and unsatisfactory reports and the improved detection of severe cytological abnormalities is shown, particularly for one GP Practice (PO) where the percentage of and U/S and B/L reports was substantially reduced.

| Cytology | All 16 GP Practices | | Practice PO | |
|----------------------|---------------------|----------------------------|-------------|----------------------------|
| | LBC | Conventional Pap ('98-'99) | LBC | Conventional Pap ('98-'99) |
| U/S | 0.6% | 10.2% | 0.4% | 7.3% |
| WNL | 92.4% | 82.1% | 96.2% | 85.4% |
| B/L | 2.3% | 4.1% | 1.7% | 4.5% |
| Mild dyskaryosis | 2.6% | 2.3% | 0.4% | 1.9% |
| Moderate dyskaryosis | 0.8% | 0.9% | 0.0% | 0.4% |
| Severe dyskaryosis | 1.3% | 0.5% | 1.3% | 0.5% |
| Other | 0.1% | 0.1% | 0.0% | 0.0% |
| TOTAL | 4702 | 8660 | 239 | 576 |

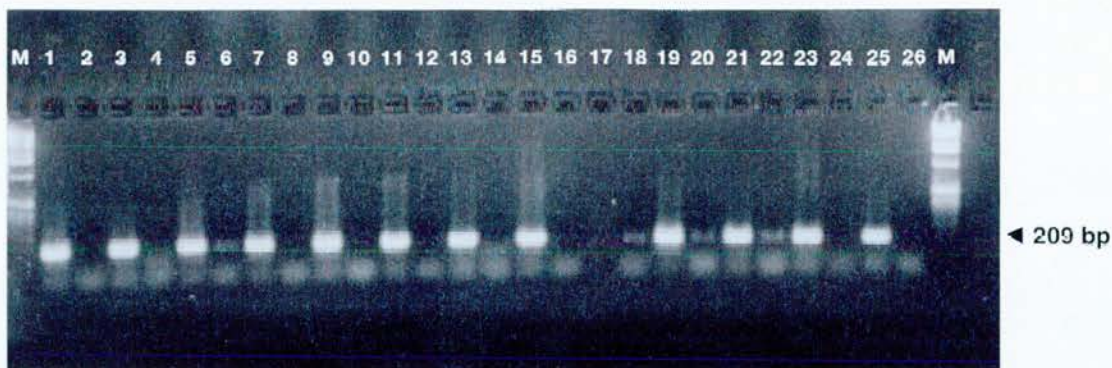
Table 7.2 Comparison of LBC and conventional Pap cytology results for all 16 GP Practices and Practice PO involved in this study

U/S = unsatisfactory; WNL = Within Normal Limits; B/L = borderline

7.4.2 Validation of BioRobot® 9604 protocol for DNA extraction from LBC specimens

During BioRobot® 9604 protocol validation, when only cell culture material was used during extraction runs, there did appear to be some weak cross-over contamination across the QIAamp® plate, as shown by β -globin PCR (Figure 7.1). Despite the use of fresh reagents, tips and extensive swabbing of the BioRobot apparatus with 70% ethanol, decontamination of the centrifuge with weak HCl and washing of the removable hardware with laboratory disinfectant (1% Virkon), this could not be eliminated. During this development stage, liquid transfer failures had often resulted in temporary stoppage of the procedure so reagents could be manually added to the QIAamp® extraction plate wells. To eliminate the possibility that this intervention was the cause of the cross-contamination, the automated extraction protocol was repeated using only Buffer ATL in the wells without manual intervention. Nevertheless, two of 24 ATL samples were still β -globin PCR positive. Weak β -globin PCR results remained in subsequent extractions using Buffer ATL only despite UV irradiation of the main BioRobot platform, all movable hardware, the centrifuge (and the buckets within) and pipettes.

GP5+/6+ PCR was also performed on the DNA extracts to determine if the contamination was occurring due to the presence of HPV DNA or other viral DNA that was present in the laboratory where the BioRobot was situated. Some weak HPV PCR positive results were obtained suggesting the source of PCR contamination was indeed occurring from the laboratory (data not shown). Thorough washing of the benches and floor around the BioRobot with soapy water eliminated the problem and subsequent β -globin and GP5+/6+ PCRs on negative control material showed no amplification. Subsequently, the BioRobot platform and surrounding bench area were washed with 70% ethanol before and after use and access to the area housing the platform was restricted.



| Gel Lane | Position on QIAamp® plate during extraction | Extracted sample | β-globin PCR result |
|----------|---|------------------|---------------------|
| 1 | A1 | SiHa cells | + |
| 2 | B1 | ATL buffer | – |
| 3 | C1 | SiHa cells | + |
| 4 | D1 | ATL buffer | – |
| 5 | E1 | SiHa cells | + |
| 6 | F1 | ATL buffer | (+) |
| 7 | G1 | SiHa cells | + |
| 8 | H1 | ATL buffer | – |
| 9 | A2 | SiHa cells | + |
| 10 | B2 | ATL buffer | – |
| 11 | C2 | SiHa cells | + |
| 12 | D2 | ATL buffer | – |
| 13 | E2 | SiHa cells | + |
| 14 | F2 | ATL buffer | – |
| 15 | G2 | Caski cells | + |
| 16 | H1 | ATL buffer | – |
| 17 | A3 | SiHa cells | Evaporated |
| 18 | B3 | ATL buffer | (+) |
| 19 | C3 | SiHa cells | + |
| 20 | D3 | ATL buffer | (+) |
| 21 | E3 | SiHa cells | + |
| 22 | F3 | ATL buffer | (+) |
| 23 | G3 | Caski cells | + |
| 24 | H3 | ATL buffer | – |

Figure 7.1 β-globin PCR results showing contamination of samples during automated DNA extraction using the BioRobot® 9604. Weak β-globin PCR positive results¹ due to contamination seen in Lanes 6, 18, 20 and 22. Lanes 25 and 26 contain the PCR positive (SiHa DNA) and negative controls (sterile water) respectively. M = kb ladder; – = PCR negative result; + = PCR positive result; (+) = weak PCR positive result

¹ As shown by presence of specific PCR product (209bp)

7.4.3 DNA Extraction from LBC specimens using BioRobot® 9604

7.4.3.1 Sample volume effect

Testing of clinical specimens collected using a fluid optimised for cell preservation resulted in problems during the automated DNA extraction procedure. Blockage of the QIAamp® extraction plate was initially attributed to insufficient sample lysis and considerable time was spent experimenting with the QIAsoft™ 3.0 software and heat-block temperatures. The heat-block temperature was difficult to record since daily fluctuations in the room temperature of the laboratory affected the ability of the heat-block to reach the correct temperature within the prescribed time. Despite attempts to improve specimen lysis by increasing the temperature or extending lysis time, a substantial number of samples were found to still cause clogging of the QIAamp® plate (Table 7.3). After extraction of 873 specimens, clogging of the QIAamp® plate was still a considerable problem with 328/873 (37.5%) of samples failing to pass through the silica membrane during the extraction process (Table 7.4).

The degree of blockage observed was related to the total volume of residual sample used for processing, with 41.8% (322/770) of samples more than 12mls in volume causing column clogging, 6.9% of samples with 9–12mls processed caused column clogging and 6.6% of samples with between 5 and 8mls of residual LBC sample processed caused blockage. No blockages were seen when 1–4mls of fluid was processed but no HPV positive results had been obtained in this group either, due to the small numbers involved. It appeared therefore that overloading the column with cells was causing the silica matrix to become blocked thus preventing efficient extraction. Insufficient specimen lysis may also be partly due to the use of a methanol-based fixative for specimen collection, which toughens any protein present thus reducing the efficiency of specimen digestion by proteinase K. Manual intervention to centrifuge the QIAamp® extraction plate often resolved the blockages, but this action compromised the use of the automated procedure.

| Number of specimens extracted | Starting volume | Lysis Conditions | Blocked Columns | | | | Unblocked Columns | | | |
|-------------------------------|-----------------|---|-----------------|-------------|-------------------|---------------------|-------------------|-------------|-------------------|---------------------|
| | | | n | % | Volume Range (ml) | Average Volume (ml) | n | % | Volume Range (ml) | Average Volume (ml) |
| 92 | 360µl | 56°C for 60 mins in Biorobot heat–block | 60 | 65.2 | 10.4–19.0 | 15.71 | 32 | 34.8 | 3.0–19.0 | 13.25 |
| 92 | 360µl | Pre-incubation at 56°C for 30 mins in water bath | 54 | 58.7 | 13.5–20.0 | 15.8 | 38 | 41.3 | 4.0–19.0 | 12.7 |
| 92 | 180µl | 56°C for 75 mins in water bath only | 12 | 13.0 | 15.0–19.0 | 16.75 | 80 | 87.0 | 4.0–20.0 | 14.94 |
| 93 | 180µl | Pre-incubation at 70°C for 60 mins in water bath and 40µl Proteinase K during 56°C lysis in Biorobot heat–block | 34 | 36.6 | 15.0–20.0 | 17.59 | 59 | 63.4 | 2.5–20.0 | 15.75 |
| 46 | 180µl | Pre-incubation at 100°C for 20 mins | 23 | 50 | 14.0–20.0 | 18.22 | 23 | 50.0 | 1.0–20.0 | 13.26 |
| 93 | 180µl | 56°C for 120 mins in water bath with mixing steps | 31 | 33.3 | 15.0–20.0 | 18.16 | 62 | 66.7 | 1.0–20.0 | 15.11 |
| 93 | 180µl | 70°C for 120 mins in water bath with mixing steps | 58 | 62.4 | 13.0–20.0 | 16.74 | 35 | 37.6 | 1.5–19.0 | 13.78 |
| 93 | 180µl | TOTAL | 64 | 68.8 | 12.0–20.0 | 17.05 | 29 | 31.2 | 3.0–18.0 | 15.24 |
| | | | 333 | 48.2 | 10.4–20.0 | 17.0 | 358 | 51.8 | 1.0–20.0 | 14.25 |

Table 7.3 The influence of different experimental conditions for specimen lysis during BioRobot® 9604 DNA extraction on the QIAamp® plate blockage rate.

| Specimen Volume | BioRobot Extraction | | | TOTAL |
|------------------------|----------------------------|-------------------------------------|-------------------------|------------|
| | Blocked Column (% HPV Pos) | Slightly Blocked Column (% HPV Pos) | No blockage (% HPV Pos) | |
| >12mls | 305 (10.2) | 17 (6.2) | 448 (11.4) | 770 |
| 9–12mls | 4 | 1 (100) | 67 (22.2) | 72 |
| 5–8mls | 1 | 0 | 14 (25.0) | 15 |
| 1–4mls | 0 | 0 | 14 | 14 |
| Unknown (T371 & T1057) | 0 | 0 | 2 (50.0) | 2 |
| TOTAL | 310 (10.1) | 18 (11.8) | 545 (13.1) | 873 |

Table 7.4 The influence of specimen volume on the QIAamp® plate blockage rate for 873 samples where the total residual LBC volume was used for specimen processing

7.4.3.2 Sample quality effect

PreservCyt® fluid was designed for optimal cell preservation for cytological assessment and cervical specimens are particularly mucous in nature. In an attempt to overcome these obstacles to DNA extraction, 177 mucus-rich specimens were treated with CytoLyt (CL) solution, which is designed to dissolve mucous in respiratory and gastrointestinal samples prior to processing. In addition, due to problems encountered with blocking of the QIAamp® membranes during the automated extraction process, the volume of sample removed from each PreservCyt® sample vial for processing was reduced to 10mls. This volume was chosen because at that point no samples with 10mls of PreservCyt® fluid processed for DNA extraction had shown blockage of the QIAamp® membranes and the issue surrounding possible loss of HPV sensitivity had not been adequately addressed. Sample processing was modified so that after centrifugation of 10mls of PreservCyt® fluid, 10mls CL solution was added to each specimen pellet before re-centrifugation as recommended by the manufacturer for processing of non-gynaecological specimens. However, the blockage rate was still found to be 23.7% (42/177; Table 7.5).

| QIAamp® plate | Cytology | n | HPV Pos (%) | HPV Neg |
|------------------------------------|----------|------------|------------------|-----------|
| Blocked columns (n=42) | U/S | 1 | NT | NT |
| | Neg | 38 | 6 (15.8) | 32 |
| | mild | 2 | 2 | 0 |
| | sev | 1 | 1 | 0 |
| TOTAL | | 42 | 9 (21.4) | 32 |
| Slightly blocked columns (n=13) | Neg | 12 | 2 (16.7) | 10 |
| | B/L | 1 | 0 | 1 |
| TOTAL | | 13 | 2 (15.4) | 11 |
| No blocked columns (n=122) | U/S | 1 | 1 | 0 |
| | Neg | 108 | 14 (13.0) | 94 |
| | B/L | 4 | 3 (75.0) | 1 |
| | mild | 4 | 4 | 0 |
| | mod | 3 | 3 | 0 |
| | sev | 2 | 2 | 0 |
| TOTAL | | 122 | 27 (22.1) | 95 |

Table 7.5 DNA extraction of 177 CytoLyt-treated specimens using the BioRobot® 9604

7.4.3.3 Effect of blockage on HPV detection

A further 668 samples were processed using only 10mls of the available sample. Of these, 25.8% (173/668) were found to cause blockage of the QIAamp® plate during extraction (Table 7.6). At this point, 50 samples were selected from 14 women who had a current smear showing some degree of dyskaryosis and 36 women who had a current negative smear but were on early recall for a previous cytological abnormality, and therefore were more likely to harbour HPV in the cervix than women with no history of cervical disease.

| Degree of QIAamp® plate blockage | HPV Pos (%) | HPV Neg | HPV NT | TOTAL |
|------------------------------------|-------------|---------|--------|-------|
| Blocked extraction column | 32 (18.5) | 139 | 2 | 173 |
| Slightly blocked extraction column | 9 (23.6) | 29 | 0 | 38 |
| No blockage | 98 (21.4) | 358 | 1 | 457 |
| TOTAL | 139 | 526 | 3 | 668 |

Table 7.6 QIAamp® plate blockage rates for 668 samples where 10mls LBC volume was used for specimen processing

NT = not tested

Reduction of the volume of processed PreservCyt® specimen from 10mls to 5mls caused less overloading of the QIAamp® membranes and thus reduced the clogging of the QIAamp® extraction plate from 5% to 1%. 8% of 10ml-processed samples caused QIAamp® plate blockage and 2% of 5ml-processed samples caused QIAamp® plate blockage. None of the 1ml-processed samples caused blockage of the QIAamp® plate during the extraction process. Twenty-one of these 50 samples (42%) produced HPV positive results by LC GP5+/6+ PCR when 10ml or 5ml volumes were used for automated DNA extraction (Table 7.7). However, only 19/50

samples produced HPV positive LC GP5+/6+ PCR results when 1ml of processed specimen was used for extraction.

This preliminary experiment using 10ml, 5ml and 1ml of sample suggested that processing 5ml of the residual original LBC specimen was optimal for reducing the QIAamp® plate blockage rate to less than 5% without any loss of sensitivity for HPV detection by real-time PCR. Additional testing of 150 specimens using the above three processing volumes confirmed these findings but the blockage rates were increased to 17.5% and 3% for 10ml- and 5ml-processed sample volumes respectively (Cuschieri *et al.*, manuscript in preparation).

| | 10mls or 5mls of LBC specimen processed for DNA extraction | | | 1ml of LBC specimen processed for DNA extraction | | |
|----------------------|--|-----------|-----------|--|-----------|-----------|
| Cytology | HPV Pos | HPV Neg | TOTAL | HPV Pos | HPV Neg | TOTAL |
| WNL | 8 | 28 | 36 | 6 | 30 | 36 |
| B/L | 6 | 0 | 6 | 6 | 0 | 6 |
| Mild dyskaryosis | 4 | 0 | 4 | 4 | 0 | 4 |
| Moderate dyskaryosis | 2 | 1 | 3 | 2 | 1 | 3 |
| Severe dyskaryosis | 1 | 0 | 1 | 1 | 0 | 1 |
| TOTAL | 21 | 29 | 50 | 19 | 31 | 50 |

Table 7.7 HPV testing results using LC GP5+/6+ PCR for pre-selected samples processed using both 10mls, 5mls and 1mls of the residual LBC specimen

WNL = within normal limits; B/L = borderline

7.4.4. HPV DNA detection using LightCycler PCR

Table 7.8 shows the cytology results and LC GP5+/6+ PCR testing results for 1082 specimens. This group comprises 300 of the first 309 specimens that had the total residual volume extracted using the manual QIAamp® tissue protocol and 782 of the subsequent 873 samples had the total residual volume extracted using the BioRobot® 9604 automated DNA extraction protocol. DNA extracted from 100 LBC samples was not tested by real-time PCR. From these results it can be seen that increasing HPV positivity was associated with increased cytological abnormality. 7.9% of women with no cytological abnormality were HPV positive, 43.5% of women with borderline cytology harboured HPV genotypes and 77.4% of women with mild dyskaryosis tested HPV positive using the real-time PCR assay. It was also possible to obtain HPV positive results in the absence of satisfactory cytology.

| TP Cytology | LC GP5+/6+ | | TOTAL |
|-----------------------|-------------|------------|-------------|
| | HPV Pos (%) | HPV Neg | |
| U/S | 1 | 4 | 5 |
| WNL | 80 (7.9) | 927 | 1007 |
| B/L | 10 (43.5) | 13 | 23 |
| Mild dyskaryosis | 24 (77.4) | 7 | 31 |
| Moderate dyskaryosis | 2 (66.6) | 1 | 3 |
| Severe dyskaryosis | 8 (66.6) | 4 | 12 |
| Glandular abnormality | 0 | 1 | 1 |
| TOTAL | 125 | 957 | 1082 |

Table 7.8 Correlation between LBC result and HPV results using LightCycler GP5+/6+ PCR for 1082 specimens that were processed for DNA extraction using the total residual LBC volume

U/S = unsatisfactory; WNL = within normal limits; B/L = borderline

Five out of 15 samples showing moderate or severe dyskaryosis were HPV negative. To eliminate the possibility of a false negative LC GP5+/6+ PCR result, these specimens were tested using conventional GP5+/6+ PCR. Four out of 5 generated HPV positive results, although two were only weakly positive. The HPV negative result was associated with a LBC specimen that had shown severe dyskaryosis upon cytological assessment. No problems had been encountered during automated DNA extraction from pelleted cells from this sample, which had 16mls of the PreservCyt® fluid processed for HPV testing.

Table 7.9 shows the cytology results and LC GP5+/6+ PCR testing results for the 668 specimens (3 were not LC tested) that had 10ml of the residual volume processed for DNA extraction. Once again, increased HPV positivity was associated with increased cytological abnormality but the degree of HPV positivity was higher in this group. 14.8% of women with no cytological abnormality were HPV positive, 68.7% of women with borderline cytology harboured HPV genotypes and all women with mild or moderate dyskaryosis tested HPV positive using LightCycler GP5+/6+ PCR. One LBC specimen that showed severe dyskaryosis was HPV negative and one LBC sample reported as adenocarcinoma was also HPV negative. No further testing was performed on either of these samples.

7.4.5. Detailed melting temperature analysis of LightCycler PCR-positive products

Detailed melting temperature analysis for HPV products showed that the majority of PCR positives were classified as HPV16 (44.3%). Several HPV18 (15.5%) and double infections (12.5%) were also found. Approximately 19% of the samples tested appeared to contain other HPV genotypes as suggested by different melting temperatures (range 76.1°C to 82.3°C), and these were designated HPV 'X'.

| Cytology | LC GP5+/6+ PCR | | TOTAL |
|----------------------|----------------|------------|------------|
| | HPV Pos (%) | HPV Neg | |
| Unknown | 1 | 5 | 6 |
| U/S | 1 | 3 | 4 |
| WNL | 89 (14.8) | 511 | 600 |
| B/L | 11 (68.7) | 5 | 16 |
| Mild dyskaryosis | 17 (100) | 0 | 17 |
| Moderate dyskaryosis | 7 (100) | 0 | 7 |
| Severe dyskaryosis | 13 (92.8) | 1 | 14 |
| Adenocarcinoma | 0 | 1 | 1 |
| TOTAL | 139 | 526 | 665 |

Table 7.9 Correlation between LBC result and HPV results using LightCycler GP5+/6+ PCR for 668 specimens that were processed for DNA extraction using 10mls of the residual LBC volume

U/S = unsatisfactory; WNL = within normal limits; B/L = borderline

7.4.6 HPV genotyping using the RLBA

The RLBA was performed to identify the HPV types present in LC GP5+/6+ PCR samples and to assess whether detailed melt analysis of LC GP5+/6+ PCR products was accurate for the identification of HPV16 and HPV18 in clinical samples. Detailed melt analysis of 117 samples gave Tms within the range 78.3°C to 79.9°C and thus were considered to be HPV16. Of these samples that were tested by the RLBA assay, 23/39 were found to contain HPV 16 DNA but 16 of these also had additional HPV genotypes present (Table 7.10). The remaining 15 samples were found to contain HPV types other than HPV16, with up to four different HPV genotypes being detected in the same sample. Specimens found to contain HPV11, 45, 56, 35 and MM9 by the RLBA assay gave similar detailed melting temperatures to the HPV16 controls used in LC GP5+/6+ PCR. Similarly, 41 samples were considered to be HPV18 on detailed melt analysis following LC GP5+/6+ PCR. Six out of 18 samples tested by the RLBA assay were found to contain HPV18 DNA, but four of these contained additional HPV genotypes also (Table 7.11). Samples containing HPV11 and HPV52 by the RLBA assay gave similar detailed melting temperatures to HPV18 control material.

| HPV Genotype assigned by Tm analysis | Total Number | Number RLBA Tested | RLBA RESULT | | Number of HPV genotypes detected other than HPV 16 | | | | |
|--------------------------------------|--------------|--------------------|----------------------|--------------------------|--|----------|----------|----------|----------|
| | | | HPV 16 only | HPV 16 + other genotypes | HPV 'X' | | | | |
| HPV 16 | 117 | 39 | 7 (mean Tm =79.2) | 16 | 2 (+1 NT) (mean Tm =78.95) | 5 | 6 | 1 | 1 |

| HPV16 + other genotypes detected by RLBA (Tm result) | Other HPV genotypes detected (Tm result) | | | |
|--|--|----------------------------------|------------------------|----------------------------|
| | HPV x1 n=5 | HPV x2 n=6 | HPV x3 n=1 | HPV x4 n=1 |
| HPV 16 + 51 (78.97) | HPV 11 (79.73) | HPV 53, 66 (78.81) | HPV 31, 58, 56 (79.41) | HPV 35, 52, 68, 54 (79.16) |
| HPV 16 + 51 + 52 x2 (79.19; 79.64) | HPV 45 ¹ (79.77) | HPV 35, 66 (78.46) | | |
| HPV 16 + MM9 x2 (79.2; 79.05) | HPV 56 (78.43) | HPV MM7, 54 (79.84) | | |
| HPV 16 + 58 x2 (78.53; 79.51) | HPV 35 (79.14) | HPV 45, 58 (79.64) | | |
| HPV 16 + 45 + 52 (78.73) | HPV MM9 (78.37) | HPV 39, 55 (78.6) | | |
| HPV 16 + 56 x2 (78.66; 79.3) | | HPV 51, MM4 ² (79.22) | | |
| HPV 16 + 33 (78.7) | | | | |
| HPV 16 + 59 (79.08) | | | | |
| HPV 16 + 31 (78.74) | | | | |
| HPV 16 + 52 + 43 (78.33) | | | | |

Table 7.10 RLBA results for samples denoted as HPV 16 by real-time PCR with GP5+/6+ primers and detailed Tm analysis, including breakdown of detailed Tm results for samples considered to be HPV 16 but confirmed to contain other HPV genotypes by RLBA analysis

n = number; Tm = melting temperature; NT = not tested

¹ In our hands, HPV 45 cloned material gave a detailed Tm of 78.2°C after GP5+/6+ LC PCR amplification (Cubie *et al.*, 2001)

² Manos *et al.*, (1994)

| HPV Genotype assigned by Tm analysis | Total Number | Number RLBA Tested | RLBA RESULT | | Number of HPV genotypes detected other than HPV 18 | | | | | |
|--------------------------------------|--------------|--------------------|-----------------------|--------------------------|--|--------|--------|--------|--------|--|
| | | | HPV 18 only | HPV 18 + other genotypes | HPV 'X' | HPV x1 | HPV x2 | HPV x3 | HPV x4 | |
| HPV 18 | 41 | 18 | 2 (mean Tm =80.55) | 4 | 4 (mean Tm =81.15) | 2 | 4 | 1 | 1 | |

| HPV16 + other genotypes detected by RLBA (Tm result) | Other HPV genotypes detected (Tm result) | | | |
|--|--|--------------------|-------------------------|-----------------------------|
| | HPV x1 | HPV x2 | HPV x3 | HPV x4 |
| HPV 18 + 16 (80.7) | HPV 11 (80.7) | HPV 56,59 (81.34) | HPV 56, MM9, 66 (80.08) | HPV 58, 53, 54, MM8 (80.27) |
| HPV 18 + 16 + 31 (80.62) | HPV 52 (81.36) | HPV 42,57 (80.69) | | |
| HPV 18+ 33+ 35+ 51+ 58+ 54 (81.17) | | HPV 6, MM8 (80.15) | | |
| HPV 18+ 16+ 52+ MM9+ 6+ 53+ 66 (81.62) | | HPV 52,53 (80.36) | | |

Table 7.11 RLBA results for samples denoted as HPV 18 by real-time PCR with GP5+/6+ primers and detailed Tm analysis, including breakdown of detailed Tm results for samples considered to be HPV 18 but confirmed to contain other HPV genotypes by RLBA analysis

n = number; Tm = melting temperature; NT = not tested

Detailed melt analysis of 51 samples gave T_m values outwith the ranges obtained using HPV 16 and HPV 18 control material. These samples were considered to contain DNA of other HPV genotypes, thus designated HPV 'X'. RLBA testing of 18 of these samples showed that eight contained more than one HPV type (Table 7.12). Four of the single infections found by the RLBA assay were HPV 16. These four samples had given detailed T_m values of 78.10, 78.19, 77.85 and 78.11. The remaining single infections were found to contain genotypes other than HPV 16: HPV 31, 58, 45 and MM9.

Fourteen of 33 samples considered to contain more than one HPV genotype by detailed melting analysis (as shown by the presence of more than one peak) were tested using the RLBA to determine the exact HPV types present (Table 7.12). Only four samples were found to contain multiple HPVs. The remaining seven samples contained either HPV 16, 18, 35, 45 or MM9.

To assess any association between the number of infecting HPV genotypes and the development of cervical abnormalities, the RLBA assay results were compared to the LBC results (Table 7.13). Infection with multiple HPV types appeared to be more common in women with normal cytology, with up to seven different HPV genotypes being detected in a single specimen. Prior infection with specific HPV types is not believed to affect the risk of acquiring another infection with a phylogenetically related type (Thomas *et al.*, 2000). Up to four HPV types were found in women with severe dyskaryosis.

| HPV Genotype assigned by T _m analysis | Total Number | Number RLBA Tested | Number of HPV genotypes detected other than HPV 18 | | | | |
|--|--------------|--------------------|--|--------|--------|--------|--------|
| | | | HPV 'X' | HPV x1 | HPV x2 | HPV x3 | HPV x4 |
| Type 'X' | 51 | 18 | | 9 | 5 | 0 | 3 |
| Double infection | 33 | 14 | | 7 | 3 | 0 | 1 |

| HPV genotypes detected by RLBA (T _m result) | | | |
|--|---------------------------------------|---------------------------|--------------------------------------|
| T _m analysis indicated double HPV infection | Type 'X' by T _m analysis | HPV x1 (n=16) | |
| | | HPV x1 (n=8) | HPV x4 (n=4) |
| Type 'X' by T _m analysis | HPV 16 x4 (78.1; 78.19; 77.85; 78.11) | HPV MM9, 42 (78.27) | HPV 31, 33, 51, 56 (76.26) |
| | HPV 31 x2 (77.69; 77.77) | HPV 39, 58 (77.08) | HPV 39, 51, 56, 40 x2 (77.84; 77.14) |
| | HPV 58 (77.86) | HPV 18, MM8 (78.25) | |
| | HPV 45 (78.22) | HPV 16, 52 (77.45) | |
| | HPV MM9 (78.01) | HPV 18, MM7 (77.61) | |
| T _m analysis indicated double HPV infection | HPV 18 x2 (80.22+80.63; 80.2+78.21) | HPV 33, MM9 (77.77+78.1) | HPV 16, 18, 58, 53 (79.24+78.64) |
| | HPV 45 x2 (79.08+77.67; 81.72+82.13) | HPV 31, 6 (79.17+78.0) | |
| | HPV 35 (78.37+78.72) | HPV 16, MM9 (78.79+79.14) | |
| | HPV 16 (77.75+78.75) | | |
| | HPV MM9 (78.7+79.22) | | |

Table 7.12 RLBA results for samples denoted as HPV 'X' or possible double HPV infection by real-time PCR with GP5+/6+ primers and detailed melting temperature analysis

T_m = melting temperature

| Cytology | Number of samples | No. of HPV genotypes detected by RLBA | | | | | | | |
|----------------------|-------------------|---------------------------------------|-----------|-----------|----------|----------|----------|----------|----------|
| | | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| Negative (WNL) | 47 | 10 (+1NT) | 17 | 14 | 2 | 1 | 0 | 1 | 1 |
| Borderline (ASCUS) | 3 | 0 | 1 | 0 | 2 | 0 | 0 | 0 | 0 |
| Mild dyskaryosis | 18 | 0 | 6 | 7 | 1 | 3 | 1 | 0 | 0 |
| Moderate dyskaryosis | 7 | 0 | 2 | 4 | 1 | 0 | 0 | 0 | 0 |
| Severe dyskaryosis | 14 | 0 | 6 | 5 | 1 | 2 | 0 | 0 | 0 |
| TOTAL | 89 | 10 (+1NT) | 32 | 30 | 7 | 6 | 1 | 1 | 1 |

Table 7.13 Correlation between LBC result and number of HPV genotypes detected by RLBA

NT = not tested

7.5 Discussion

7.5.1 General problems associated with automated DNA extraction from LBC Specimens

To perform HPV testing on residual LBC fluids, a suitable DNA extraction procedure is necessary that can successfully extract nucleic acid from a large number of specimens. This proved to be a difficult task since PreservCyt® fluid is an methanol-based medium designed to preserve cell morphology. Since the number of HPV-infected cells in any LBC specimen may be small, a main concern of this study was that reducing the volume of sample processed would compromise the sensitivity of the downstream GP5+/6+ LC PCR assay for the detection of HPV DNA. Standardisation of LBC specimens would be very difficult since the nature of individual specimens (for example, mucous or blood content) and the amount of residual fluid remaining after the LBC smear had been prepared differed from specimen to specimen. Ideally, quantification of the number of cells in each LBC sample would have allowed standardisation of the extracted material. This could have been done by quantitatively estimating cellular β -globin. For instance, quantifying the number of cells per specimen would have allowed an exact volume of PreservCyt® fluid to be processed containing a known number of cells e.g. 10^6 cells/ml. After resuspension of the cell pellet in 180 μ l Buffer ATL, every specimen that was extracted would have contained the same number of cells. However, this practice does not control for the proportion of HPV-infected cells per sample. Since cell quantification would be very labour intensive, it was not considered suitable for high specimen throughput, and thus was not investigated as part of this study.

Instead, LBC specimens were processed using varying volumes of the residual LBC specimen and DNA extraction experiments were performed using the automated BioRobot® 9604 protocol. When the entire residual LBC specimen volume was processed for extraction, approximately 38% of the QIAamp® extraction plate columns were blocked. Pre-treatment of the residual LBC fluid with CytoLyt solution (designed to break down mucous in respiratory samples) and attempts to improve the specimen lysis stage did not improve the QIAamp® plate blockage rate.

Blockage of the QIAamp® extraction plate required manual intervention: additional QIAamp® plate centrifuge steps at 10,000rpm were performed to remove blockages since centrifugal force was more effective than a vacuum at drawing the samples across the QIAamp® silica membrane. After QIAamp® extraction plate blockage, subsequent washing steps were often also problematic and manual intervention to centrifuge the plate was again required to resolve the blockage problems. These additional centrifuge steps were time-consuming and compromised the automated advantage of using the BioRobot® 9604. Reduction of the processed LBC specimen volume to 10ml did reduce the blockage rate to 23.7% but this was still considerably higher than the 5% blockage rate considered acceptable by the BioRobot® 9604 manufacturer (QIAGEN Ltd., personal communication). If <5% blockages occur, the BioRobot® 9604 can be programmed to locate the blocked sample(s) on the QIAamp® extraction plate and remove the sample from the plate well. Meanwhile, the software notes the blocked sample and the extraction procedure continues without interruption.

Additional investigations performed on 50 selected LBC specimens using 10ml, 5ml and 1ml volumes of the original sample for processing showed that reduction of the processed LBC specimen volume to 5ml resulted in a QIAamp® extraction plate blockage rate of <5%, without a subsequent loss in sensitivity for the detection of HPV by GP5+/6+ LC PCR. Using 1ml of residual LBC specimen for processing did not cause any blockage of the QIAamp® extraction plate columns during the automated protocol but reduced sensitivity was found for the detection of HPV DNA by GP5+/6+ LC PCR since two samples that tested HPV positive when 5mls of fluid was used for extraction, tested HPV negative when only 1ml was used. Thus, 5mls of PreservCyt® fluid was recommended as the volume to be processed for automated nucleic acid extraction and any QIAamp® plate blockages that occurred were noted and dealt with by the BioRobot® 9604 automatic well-clearance facility.

Due to the large number of LBC specimens received in a six-month period (approximately 250–300 specimens per week) and the problems encountered during development of the automated DNA extraction protocol, many specimens were

processed some time before either DNA extraction or GP5+/6+ LC PCR testing was performed. Thus, in most cases, retrospective DNA extraction and LC PCR testing were performed after cells from the residual specimen volume had been pelleted. Thus many of the specimens had already been processed using a 10ml sample volume before the influence of this sample volume on QIAamp® plate blockage rates was detected.

7.5.2. Technical problems associated with DNA extraction from LBC specimens using the BioRobot® 9604

Initial work to optimise the BioRobot® 9604 extraction protocol was complicated by cross-contamination occurring across the QIAamp® extraction plate. Considerable time and effort was spent investigating this. Following helpful discussion with Drs Jon Turner and Shona Kerr at the Molecular Medicine Centre, Western General Hospital, Edinburgh, who have expertise in high-throughput DNA extraction from clinical specimens, the location of the BioRobot was deemed the most likely explanation for the PCR contamination problem. Due to the small area dedicated for DNA extraction at RCVL, the BioRobot had been assembled in the main molecular laboratory, which has designated areas for LBC specimen processing, PCR amplification and agarose gel electrophoresis. It was thought that β -globin PCR positivity was due to PCR product contamination of the BioRobot bench area. Use of another primer set for the detection of another cellular gene (kindly provided by Dr. J. Warner) failed to generate any positive results (data not shown), thus confirming that PCR product was the explanation for negative control contamination.

Other problems encountered with the BioRobot protocol development included software problems such as liquid detection errors, dripping from the tips, total sample transfer from the heat block to QIAamp® plate and software failures. Due to the initial problems with PCR product contamination of the BioRobot® 9604 platform and bench area, the instrument and bench tops were swabbed down with 70% ethanol before and after use. After each extraction run the centrifuge was wiped using weak acid then water, the vacuum manifold was washed thoroughly in 1% Virkon disinfectant then rinsed with water and the plastic square-well blocks were

autoclaved. These measures were performed to minimise the risk of PCR product contamination.

Although the BioRobot® 9604 extraction method could process 96 samples in one run, it took approximately 3.5 hours to complete the protocol. The limiting step was the plate centrifugation stage (to dry the QIAamp® plate prior to elution) that prevented the extraction procedure being completely automated and a true ‘walk-away’ system.

7.5.3. LightCycler PCR

Real-time PCR offers many advantages over conventional PCR systems. Since real-time GP5+/6+ PCR amplification and product detection occur simultaneously in the same glass capillary, post-PCR manipulations are unnecessary. Hence, this method is much quicker and less labour intensive than conventional GP5+/6+ PCR methods. In the study presented here, the entire DNA extraction and amplification procedure for 96 samples could be performed within approximately 4.5 hours. In contrast, the time required for manual DNA extraction of 24 samples is at least one hour, followed by a 4-hour conventional PCR amplification step then 30 minutes for detection of the PCR product by agarose gel electrophoresis; a minimum of 5.5 hours. The true strength of real-time PCR is the ability to quantitate the amount of target DNA present. This is important because some studies have indicated that the quantity of HPV DNA detected is predictive of cervical disease (Ylitalo *et al.*, 2000; Josefsson *et al.*, 2000). Although quantitative analysis of clinical samples was not included in the present study, extraction of HPV DNA from a known copy number of SiHa cells shows that the LightCycler could be used for this purpose. We found that 10-fold dilutions of SiHa and HeLa cells of known copy number for HPV16 and HPV18 could be detected as parallel amplification curves, approximately three cycles apart (data not shown). Quantitative fluorescence-based real-time PCR assays have been developed by others, particularly for cytomegalovirus – a major cause of morbidity and mortality in immunosuppressed individuals such as HIV patients and solid organ transplant recipients (Schaade *et al.*, 2000; Kearns *et al.*, 2001; Gault *et al.*, 2001).

Practical problems encountered in the study reported here included handling the fragile capillaries and interpreting the LC PCR T_m analysis results. Some samples produced a very low melting peak, which is associated with a smaller number of target copies (Schalasta *et al.*, 2000b), making visual interpretation of results more difficult. Improvements to the current system could be made by introduction of an internal amplification control to allow quantification of the input sample DNA and to detect PCR inhibitors. The LC is equipped with other channels for the detection of other reporter dyes, which could be used to introduce an internal control. For example, human β -globin is present in one copy/cell and primers for β -globin detection have been successfully incorporated into multiplex real-time PCR formats (Tucker *et al.*, 2001). Using standard curve analysis, the number of HPV copies per microgram of input DNA can then be calculated.

7.5.4. Comparison of HPV DNA detection by LightCycler PCR with T_m analysis and RLBA

Attempts were made to differentiate between HPV16, 18 and others (designated HPV 'X') using T_m analysis. Further HPV genotyping using the RLBA (that can detect up to 13 different HPV types) showed that clinical samples containing multiple HPVs are common and these could not be distinguished by detailed melt analysis of LC GP5+/6+ PCR products alone. Cervical samples from 19 out of 46 (41.3%) women with normal cytology were found to contain more than one HPV genotype (with up to seven different HPV types being detected). Such findings could lead to a requirement for HPV genotyping to increase the specificity and delineate further those HPV types most frequently associated with persistence and cervical disease progression. Different line blot assays have been reported for this purpose (Gravitt *et al.*, 1998; Kleter *et al.*, 1999; van den Brule *et al.*, 2002).

Nonetheless, the results obtained by real-time PCR detailed melt analysis and the RLBA should be interpreted with caution. These molecular assays employ different primer sets (GP5+/6+ and MY09/11 respectively) for the amplification of a wide range of anogenital HPV genotypes. It is being increasingly recognised that the technical parameters of available detection systems may have led to preferential

detection of some types and underestimation of the prevalence and thus significance of certain other HPV genotypes (J. Kornegay, personal communication). For example, GP5+/6+ primers were designed to have the least number of mismatches with HPV16 and HPV18 L1 DNA sequences. Thus, the analytical sensitivity of the GP5+/6+ PCR system will be greater for these HPV genotypes. For other HPV types, a greater number of primer mismatches result during PCR amplification and therefore DNA from such types will be amplified with less efficiency. It is noteworthy that in comparisons of the GP5+/6+ and MY09/11 primer sets for the detection of HPV DNA in cervical specimens, the MY09/11 primers were far more effective at detecting samples with multiple HPV types than GP5+/6+ (90% and 47% respectively; Qu *et al.*, 1997). Tucker and colleagues (1993) investigated whether PCR amplification of HPV DNA in cervical specimens accurately reflected the relative amounts of each HPV genotype present. They concluded that the selective amplification of one HPV over another could occur when multiple genotypes are present. It could therefore be possible that the GP5+/6+ primers are amplifying the predominant HPV genotype only during real-time PCR. The GP5+/6+ PCR system using the LightCycler was standardised using HPV16 and HPV18 cell culture material and plasmid DNA, including mixtures of these DNA types.

More work is required to optimise the LightCycler GP5+/6+ PCR system for the detection of other HPV genotypes. Since it appears impossible to distinguish between all the known HPVs by detailed melting temperature analysis alone, increased specificity may be achieved using LC hybridisation probes. This would involve fluorescence resonance energy transfer (FRET) technology through the use of two independent single-labelled oligonucleotides that are designed to hybridise adjacently on the PCR amplicon internal to the flanking PCR primers. The upstream probe would be labelled at its 3'-end with a donor dye (e.g. fluorescein) and the downstream probe would be labelled at its 5'-end with an acceptor dye (e.g. the cyanine dye Cy5TM or LightCycler Red 640). After light excitation by the LED, FRET would occur from the donor to the acceptor dye resulting in an increase in fluorescence, which would be directly proportional to the amount of PCR product generated. Real-time quantitative PCR using FRET technology has been applied for

CMV (Nitsche *et al.*, 1999; Schaade *et al.*, 2000) and HSV (Espy *et al.*, 2000b). Although the use of LC hybridisation probes might be considered more complex for HPV detection due to the larger number of viral genotypes found in clinical specimens, molecular beacons containing a hairpin loop structure in which fluorescent donor and quencher molecules are held in close proximity have been successfully applied for real-time HPV PCR systems (Jordens *et al.*, 2000; Szuhai *et al.*, 2001).

7.6 Conclusion

This was the first UK study to report on the use of LBC to replace conventional smears within the NHS Cervical Screening Programme. Monolayer preparations from PreservCyt® specimens reduced the inadequate smear rate from around 10% to less than 1%. This reduced the level of anxiety in women and provided savings in repeat visits for patients, repeat laboratory tests and administrative time. LBC also reduced the number of B/L smear diagnoses and increased the detection of severe cytological abnormalities confirmed by histology. The GPs involved in this study were very impressed by the LBC screening system and the improved results made them reluctant to return to conventional Pap screening.

New technologies to improve the cervical screening programme include detection of high-risk human papillomavirus types to identify those women at increased risk of cervical disease progression. A protocol suitable for such testing has not yet been determined, but the limitations of current hybridisation methods and consensus PCR methods for screening purposes are known. For HPV testing within cervical screening programmes, the development of an efficient, sensitive and automated extraction procedure is a prerequisite for high sample throughput. Our priority was to develop a robust system for this purpose without compromising the sensitivity of subsequent HPV testing using LC GP5+/6+ PCR. A robot extraction procedure using a QIAGEN BioRobot® 9604 was successfully developed. This method was based on cellular lysis, followed by binding of nucleic acid to a silica matrix and subsequent elution of the DNA. The manual QIAGEN extraction protocol, based on the same principle, had been successfully used for DNA extraction from LBC samples in a previous study (described in Chapter 6). However, the manual procedure used a water bath set at 55°C to perform lysis of samples contained in 1.5ml eppendorf tubes, followed by the use of centrifugal force to draw the samples across the silica membrane. The automated BioRobot® 9604 extraction protocol, used a heat block to lyse the samples that were contained in a plastic square-well block and vacuum pressure was used at most stages to draw the samples across the silica membrane. This was found to be less effective than centrifugation, resulting in a large number of

QIAamp® plate blockages. In addition, problems were encountered with mucous-rich specimens as PreservCyt® fluid is designed for optimal cell preservation, rather than DNA extraction. Pre-treatment with CytoLyt solution and improvements to the sample lysis stage of the extraction protocol failed to reduce the number of blockages, which were finally attributed to cellular overloading of the QIAamp® columns. Reducing the volume of specimen processed for DNA extraction to 5mls resulted in <5% blockages which could be dealt with by the BioRobot® 9604 and did not compromise the sensitivity of HPV detection by real-time PCR.

Although PCR allows faster result turn around times within a diagnostic laboratory, detection of conventional PCR positive samples has to be performed by agarose gel electrophoresis. LightCycler PCR eliminates the need for this extra manipulation since PCR positive samples are identified as a peak during T_m analysis. Additionally, detection of individual HPVs in conventional PCR positive samples requires testing with an enzyme immunoassay using many type-specific probes. LC GP5+/6+ PCR was found to be a rapid and efficient method for the detection of HPV in LBC extractions. It was possible to test up to 150 samples per day using one LightCycler following robotic extraction of the DNA. Since the melting temperature of DNA is dependent on the sequence, length and GC content, preliminary work using DNA extracted from cell culture material and plasmid DNA had suggested it was possible to differentiate between HPV16 and HPV18 by DNA melting temperature analysis following GP5+/6+ LC PCR. It was hoped that T_m analysis would also allow further differentiation between other HPV types. However, only 17.9% and 11.1% of single HPV16 and HPV18 infections indicated by detailed melt analysis of DNA extracted from clinical specimens were confirmed by the RLBA. RLBA testing showed that multiple HPV infections were common, both in women with and without cytological abnormalities. Apart from the suggested association of HPV18 with rapid progression of cervical neoplasia, there is yet little evidence to suggest that the risk of developing cervical cancer is different for the other high-risk HPV genotypes. It has therefore been suggested that tests that detect all the known HR-HPV types together are sufficient for clinical management. HPV genotyping is necessary to distinguish persistent HPV infection and re-infection with different

HPV types. RNA analysis may be necessary to distinguish which HPV genotypes are actively replicating and those which are latent infections. If current clinical research suggests that HPV genotyping is necessary for making clinical management decisions, then genotyping assays will be required to differentiate specific HPV types and even variants of the same type (Mayrand *et al.*, 2000).

The development and optimisation of the automated extraction system and real-time PCR system was more time consuming than expected. Of the 4702 LBC samples collected and processed over a six-month period, 1900 had DNA extracted and stored. 1750 of the 1900 samples were tested for HPV DNA during this time. GP5+/6+ PCR using the LightCycler following automated BioRobot® 9604 extraction detected HPV DNA in 90–100% of LBC samples that had shown moderate or severe dyskaryosis on cytological assessment, leading to the conclusion that the combination of automated DNA extraction using a BioRobot® 9604 and real-time LightCycler PCR is suitable for HPV detection in a diagnostic laboratory setting. Further work is required to confirm the real-time PCR findings using the RLBA assay and to optimise the real-time PCR assay for the detection of other HPV genotypes. This approach is likely to involve the use of hybridisation probes since it is impossible to distinguish between individual HPV types from the array of known genotypes on the basis of melting temperature analysis alone. This study has generated a unique resource of more than 5000 fully characterised LBC samples from consenting women attending routine cervical screening clinics within the Scottish Cervical Screening Programme. This collection will be a valuable resource for continued development of molecular tests for HPV and for other microbes infecting the female genital tract.

CHAPTER 8

GENERAL DISCUSSION

The work covered in this thesis focuses primarily on the role of HPV testing in cervical screening to improve the identification of women who are at risk of developing cervical neoplasia. To assess this, several different patient populations were investigated using various methods for HPV DNA detection. In addition, the assessment of LBC for the improved detection of cytological abnormalities was performed and the suitability of preservative fluids for HPV tests was critically evaluated. However, the potential uses of HPV testing in the cervical screening programme remains a complex issue and this chapter sets out a summary of the findings of the work that has been presented with discussions of the advantages and disadvantages on screening protocols along with description of some topics for future research that would supplement the findings.

8.1. Limitations of the current NHS cervical screening programme using the conventional Papanicolaou smear

For effective screening of cervical neoplasia, a truly representative sample taken from the cervical transformation zone is essential for assessment. Since the introduction of the Pap smear by George Papanicolaou in the 1940's, organised screening programmes have successfully reduced the mortality associated with cervical cancer. Now, the level of success achievable using the Pap smear has been reached and the drawbacks of the technique are well recognised (Gay *et al.*, 1985; van der Graaf and Vooijs, 1987). Poor preparation and fixation is a major limitation that results in a large number of inadequate and borderline Pap smear results. A significant proportion of the collected cervical cells are not transferred to the glass slide and are instead discarded along with the sampling device. Moreover, the cells that are successfully transferred are often overlapping and obscured by inflammation or blood (McGoogan and Reith, 1996). Unsatisfactory smears result in a need for repeated cytological assessment and the majority of B/L smear reports are of no

clinical significance (Cuzick *et al.*, 1999b). So, new methodologies are required to improve the sensitivity and specificity of the National Cervical Screening Programme.

8.2 The Way Ahead

8.2.1 LBC

The results presented in this thesis suggest that LBC has the potential to improve cervical cytopathology through the collection of a more representative specimen. TP slide preparations made using automated technology operated by skilled staff show optimal cell preservation as well as reduced debris from inflammatory cells, blood or mucus. Additionally, only a proportion of the cervical cells collected into the preservative fluid are used to make a TP slide, so the residual material can be processed for additional laboratory tests without another patient visit. Comparative assessment of 800 TP smears and conventional Pap smears prepared during the 'split-sample' study (Section 6.3.1), where a Pap smear was prepared in the standard way before the cells were collected into PreservCyt® fluid, showed that LBC provided a more accurate assessment of cervical cytological abnormality. While 87.5% of the smears showed complete diagnostic agreement, the TP smear detected a more severe abnormality in over half of the remaining cases. The remaining discrepant results were due to overdiagnosis of the cytological abnormality using the conventional Pap smear. Furthermore, TP significantly reduced the number of inadequate and B/L smear diagnoses (from 7.3% to 2% and from 7.9% to 4.2% respectively). The few inadequate TP reports primarily resulted from insufficient epithelial cells on the slide rather than inflammation, blood or drying artefacts. This improved effectiveness of screening was further accentuated in the 'direct-to-vial' study (Chapter 7), which was the first UK study where the TP slide replaced the conventional Pap smear for screening purposes. As a result of the entire cervical sample being collected into preservative fluid, the number of inadequate TP smears was reduced from 10% to less than 1% and the number of B/L smears was reduced from 4.1% to 2.3%. The use of TP slide preparations was again associated with greater detection of high-grade cervical abnormalities from 1.4% to 2.1%. Although

a report by the NICE in June 2000 concluded that there was insufficient evidence to support the immediate introduction of LBC, the Lothian GPs involved in the direct-to-vial study were so impressed by the clinical value of LBC that they were reluctant to return to conventional Pap screening. The analysis of data collected from the LBC demonstration projects performed at four different Scottish sites over a six month period during 2001 was reported in January 2002. Following the success of these pilot studies, the Scottish Executive have announced that £2.75 million will be invested to introduce LBC throughout Scotland for all women by 2004 (internet citation: http://www.scotland.gov.uk/pages/news/2002/03/p_SE5603.aspx).

From a laboratory perspective, the cost of non-recyclable consumables, reagents and hardware is more expensive for LBC compared to conventional Pap smears and cytotechnologists need to be retrained to interpret LBC smears. However, trained personnel can screen LBC slides in half the time taken to screen conventional Pap smears. As the time taken for administration is the same, the daily workload can be increased by 25–40% (Dr. E. McGoogan, personal communication) which is likely to have cost benefits for the National Health Service. Practical problems associated with producing LBC preparations may include the shelf-life and storage of supplies in GP clinics, education of smear-takers in the correct method for LBC sampling and the cost and practicalities of transport of alcohol-containing specimens. However, these minor drawbacks are heavily outweighed by the benefits to both the patient and clinician and the potential to generate long term savings through a reduction in the number of unsatisfactory and borderline smear reports generated. As a result, patient anxiety would also be reduced which may result in a decline in the number of women who are lost to follow-up.

8.2.2 HPV Testing

8.2.2.1 Within the CSP

The epidemiological evidence from the literature clearly shows that HPV infection is an essential element in the development of cervical neoplasia and infection with HR-HPV types is the major risk factor for the subsequent development of cervical

carcinoma, although the exact mechanism of HPV-associated cervical carcinogenesis is still incompletely understood. In the work reported here, increased HPV positivity was shown to be associated with increased cytological abnormality, with 90–100% of samples showing high-grade dyskaryosis by LBC or conventional Pap smear being found to contain HPV DNA and more than 80% of low-grade abnormalities were also HPV positive.

Although there is substantial evidence in the literature to suggest that HPV testing would be useful as an adjunct to cervical cytology, there is still debate as to whether this should be implemented for detection of cervical abnormalities as part of the primary screening process or only for management of women with B/L smear results. The results presented here suggest that testing for high-risk HPV genotypes may confer a positive role at the secondary screening stage when used in combination with LBC. Specifically, its application could allow more effective triage of women with B/L smears by delineating them into one of three groups:

- (i) return to routine screening
- (ii) continued cytological follow-up or
- (iii) immediate referral to colposcopy.

If all HPV negative women in the ‘split-sample’ study reported here (Section 6.3.7) had been managed by routine recall, then two cases of CIN 1 would have been missed but no cases of CIN 2/3 would have been left untreated. Yet 110 women would have been saved a total of 160 unnecessary repeat visits and smears. Thus HPV testing in this situation would most likely prove to be cost-effective through the reduction in colposcopy referrals. The high negative predictive value (NPV) of a negative HCAII result and normal cytology was also shown by retrospective analysis of archival Pap smears from women with a previous cervical biopsy (Section 4.4.4). Although the positive predictive value (PPV) of combined cytology and HPV testing before biopsy was higher at 78% than the PPV of cytology alone or HPV testing alone, the NPV of a negative HCAII result and normal cytology was 100%. This would support the introduction of HPV DNA testing to aid exclusion of severe

cervical disease rather than predicting its presence. In March 2001, large-scale trials of HPV testing in combination with LBC began at three separate locations in England as part of a 12-month pilot study.

Results from the work described here (Section 6.3.5) suggest that HPV testing by HCAII is unlikely to be cost effective for primary cervical screening since on average 15% of women with no previous history of cytological abnormality were found to harbour HR-HPV types in their cervixes. Since only a small proportion of these women would develop cervical neoplasia, cytological and histological follow-up of such HPV-positive cases, as part of the primary screening process, would not be cost effective. Another important consideration for the implementation of HPV testing as part of primary screening would be the age range for screening since age appears to be an important factor in the complex relationship between the sensitivity and specificity of HPV testing. In all women enrolled the split-sample study (Chapter 6), HPV positivity was found to decrease with age with the majority of HPV positive results (43.8%) obtained from women aged 24–29 years (data not shown). Evidence from the literature suggests that HPV testing is unlikely to be cost effective in women under 35 years of age since the majority of these women would only have transient viral infection. Indeed, in the split-sample study, nearly 39% of women under 35 years of age were HPV positive compared to 15% of women aged 35 or more. Only one woman over 60 years of age showed significant cytological abnormality (severe dyskaryosis) and was HPV positive. Currently, standard practice is to screen women until they are 65 years old but the results presented here suggest that older women who lack HPV do not require such prolonged surveillance. This would also have beneficial cost implications for the service.

8.2.2.2 In HIV-seropositive women

In the longitudinal study of HIV-associated HPV infection, HR-HPV positivity increased to 44.2% although this was not necessarily associated with the progression of cervical disease since the majority of abnormalities found were low-grade. Indeed, HR-HPV genotypes were found in 62.5% of HIV-seropositive women without any associated cytological abnormality. Although there are currently no

defined UK guidelines on cervical screening in this population, it would appear that cervical neoplasia progression has been contained in the Edinburgh HIV cohort by regular monitoring of patients and prompt, effective treatment of cervical lesions with good follow-up. Evidence to support this view is the observation that HPV status was not used to influence the clinical management of these patients and cases of CIN began decreasing several years ago, well before the introduction of antiretroviral drug therapy. Therefore HPV testing may be of limited use in the gynaecological management of cervical neoplasia in HIV-infected women, whereas improved patient monitoring may play a more important role in the prevention of cervical neoplasia in this high-risk group. Understandably, in some areas, achieving this may be difficult if clinic attendance rates are low. Although it is too early to assess fully if HAART will have a positive effect on cervical neoplasia progression the results reported here suggest that women with CD4 counts <200 cells/ μ l or HIV RNA viral loads of >5000 copies/ml are certainly at greatest risk of cervical neoplasia. Indeed the European MACH-1 study, which includes the Edinburgh cohort, may provide important information on the effect of antiretroviral therapies on cervical neoplasia in a larger group of HIV-infected women. Its findings will not be available for a further 2–3 years.

Caution should be applied in the clinical use of HPV testing, particularly when interpreting single time point results, even in relation to cytology. HPV tests should be performed at regular intervals, for example six-monthly, for identification of persistent HPV infection that is known to be associated with a higher risk of developing cervical neoplasia. This would increase the cost of introducing HPV testing as a primary screening tool. However, care must be taken about the use of the term ‘persistent HPV infection’. 70% of the Edinburgh HIV cohort who had at least three cervical specimens collected at approximately six-monthly intervals were shown to have HR-HPV DNA regularly detected in all samples, but the HPV types detected in the cervix changed over time in a number of cases. Thus continued detection of HPV DNA in two consecutive samples could occur as a result of lasting infection with the same HPV type, elimination of one HPV genotype and reactivation of another latent HPV infection or re-infection with a new type or variant. Direct

DNA sequence analysis of GP5+/6+ PCR products identified 22 different HPV genotypes in the Edinburgh cohort of HIV-seropositive women, including some novel types which, to date, have not been fully characterised. For example, CP8304 was shown to be associated with histologically confirmed CIN, and this has led to collaborative work with Dr E.M. deVilliers at Deutsches Krebsforschungszentrum, Heidelberg, Germany to characterise this isolate further (Dr C. Cuschieri, personal communication). It is therefore logical to hypothesise that the prevalence and oncogenic potential of some rare HPVs has probably not been fully recognised.

8.2.2.3 Usefulness of different HPV tests

The introduction of HPV testing for triage alone will result in a large number of HPV tests (approximately 250,000 in the UK). Although the format of current HPV tests would be acceptable for this volume, the introduction of HPV testing in primary screening would generate many more tests (approximately 3.5 million in the UK) (Davies *et al.*, 2001). Thus, automated DNA extraction protocols and a rapid molecular test for the detection of HPV DNA would be required to cope with the large numbers involved. However, the ability to be able to distinguish between individual HPV genotypes in a single assay and quantify the amount of DNA present would be an advantage.

Most of the studies described here have involved comparison between two of the most well-established methods for HPV DNA detection: HCAII and consensus GP5+/6+ PCR. HCAII is a simple, reproducible and user-friendly test available from Digene that can distinguish between 5 low-risk and 13 high-risk genotypes by the use of group-specific oligonucleotide probe pools for DNA detection. Use of a simple procedure (using a Digene Specimen Conversion Kit) renders PreservCyt® LBC specimens suitable for HCAII testing. However, HCAII alone cannot quantitate the amount of viral DNA present nor can it identify the specific genotype(s) present. From DNA sequence data generated as part of this work, it appears that the high-risk HCAII probe cocktail allows cross detection of some additional HPV types, particularly rare genotypes, despite the lack of specific probes for these types in the probe pool. This has been noted by others (Peyton *et al.*, 1998; Vernon *et al.*, 2000; Schopp *et al.*, 2001). Several studies have attempted to measure HPV viral load

using the relative light unit (RLU) indices produced by Hybrid Capture technology since it has been suggested that productive viral infection, as measured by high HPV viral load, is indicative of an increased risk of the development of cervical disease. However, the HCAII test involves signal amplification and was not designed for accurate DNA quantification. So, at best, comparison of RLU index values against a single standard is semi-quantitative. A standard curve with several standards of different HPV copy number would need to be included in every run for a more quantitative approach.

Conventional GP5+/6+ PCR sample preparation and final detection using agarose gel electrophoresis does not lend itself readily to automation, and therefore is very laborious for large specimen numbers. It is also impossible to differentiate between different HPV genotypes by agarose gel electrophoresis of GP5+/6+ PCR products since the amplified products are very similar in size. Genotyping must be performed by some additional method such as an enzyme immunoassay using a range of type-specific probes, restriction enzyme analysis or DNA sequencing. Results described here show that HPV positivity was higher using GP5+/6+ PCR compared to hybrid capture technology, but this technique will detect both high-risk and low-risk HPV genotypes in the same reaction. However, detection of HPV DNA by PCR is dependent on prior isolation of the DNA from infected cells. Depending on the specimen type, several approaches have been used for DNA extraction, ranging from simple boiling to phenol-chloroform extraction. DNA extraction from LBC specimens was complicated by the fact that PreservCyt® fluid is designed for optimal cell preservation rather than nucleic acid extraction and cervical scrape specimens can be particularly mucous in nature. In the work described here, manual DNA extraction using individual QIAGEN spin columns under centrifugal force was successfully used to isolate DNA from residual LBC fluid but this procedure was considered too laborious for high specimen through-put. Thus, a BioRobot® 9604 (QIAGEN Ltd.) protocol was developed for automated DNA extraction from routine LBC specimens. However, vacuum pressure was less effective than centrifugation at drawing clinical samples across the silica membrane and 37.5% of samples blocked the QIAamp® extraction plate when cells from the total residual volume were used.

Blockage of the QIAamp® extraction plate was attributed to cellular over-loading of the columns so to improve extraction efficiency, the residual volume used for processing was reduced to 5ml. This reduced the blockage rate to less than 5% without compromising downstream HPV testing. These technical improvements took considerable time for optimisation but it is hoped that QIAGEN will incorporate the final protocol in future product inserts. For large-scale HPV DNA detection, a real-time GP5+/6+PCR system was developed using the LightCycler instrument. Of the 1750 LBC specimens extracted using the automated BioRobot® 9604 extraction protocol, 15% were found to contain HPV DNA by real-time PCR including >80% of samples associated with moderate or severe cytological abnormalities.

Real-time PCR technology is beginning to revolutionise the potential of PCR by using rapid temperature control to reduce testing time (typically 40 cycles in 25 minutes) and minimising the risk of contamination by performing both PCR amplification and product detection simultaneously in closed glass capillaries. Although the glass PCR capillaries are more expensive than eppendorf tubes, the overall costs are less due to the smaller volumes of reagents used. Initial studies using the LightCycler and GP5+/6+ PCR were published recently (Cubie *et al.*, 2001). Detailed T_m analysis was used to differentiate between HPV16 (mean $T_m=79.1 \pm 0.8^\circ\text{C}$) and HPV18 (mean $T_m=80.9^\circ\text{C} \pm 1.0^\circ\text{C}$) sequences. In the work presented here, T_m analysis suggested that many of LightCycler PCR positives were HPV16 (44.3%). A further 15.5% were identified as HPV18, 12.5% contained more than one HPV genotype and 19.3% had other HPV types present (HPV 'X'). To confirm these results, a RLBA (currently available for research purposes only) was used. However, only 17.9% of single HPV16 and 11.1% of single HPV18 infections were confirmed. On-going studies using the RLBA performed at the Regional Clinical Virology Laboratory suggest that multiple HPV infections are common in women irrespective of cytology (Dr. C. Cuschieri, personal communication). It appears that T_m analysis is not sufficient to distinguish reliably between HPV 16 and HPV18 genotypes or identify multiple infection. However, these results should be interpreted with caution since both these molecular assays employ different primer sets and evidence from the literature suggests that preferential detection of some

types may result depending on the primer set chosen. For example, the analytical sensitivity of GP5+/6+ is considered to be greatest for HPV16 and HPV18 since the primers were designed to be optimal for these common genotypes whereas MY09/11 primers are more efficient at amplifying multiple HPVs (Qu *et al.*, 1997). This may explain why detailed melt analysis of 158 samples suggested that either HPV16 or 18 was present but 20/56 (35.7%) tested using the RLBA contained these and additional genotypes. It is possible that selective amplification of one genotype (i.e. the predominating HPV 16 or 18) over another was occurring during LC GP5+/6+ PCR of multiple infection samples, and only the predominant type was detected. Nonetheless, results from RLBA testing support other recent findings that the range of HPV genotypes and their association with cervical disease may be just as variable in immunocompetent women as in immunosuppressed women. At present, gynaecological management of women based on their HPV status would be independent of the associated HPV genotype(s) involved, although in future HPV detection methods may be used to identify individual HPV genotypes. The RLBA is a useful tool for PCR product analysis, since it can detect up to 27 different HPVs, including some novel HPV genotypes. A more recent typing procedure for GP5+/6+ PCR products can detect up to 37 different mucosotropic HPVs (van den Brule *et al.*, 2002). The ease of use and absence of expensive detection equipment required for such tests may make them the method of choice for HPV genotyping in the future, although the results obtained from different genotyping systems may also differ slightly due to the primers sets used (van Doorn *et al.*, 2002).

8.3 Final Conclusions and Future Work

The improved detection of cervical cytological abnormalities by the use of LBC have been very encouraging and it is anticipated that the level of improvement in inadequate smear rates will be maintained in the larger Scottish and English LBC demonstration projects. Although rigorous cost-benefit analyses are not yet complete, the future of the National Cervical Screening Programme is likely to include LBC which would result in improved detection of women who are at risk of developing significant cervical neoplasia. Rationalisation of the screening

programme by the introduction of LBC would minimise the financial implications of establishing a system of HPV testing, particularly if performed by a centralised facility designed for efficient large-scale HPV testing.

The value of HPV testing in population screening or for management of women with borderline smears is being assessed in various multi-centre studies. The results presented here suggest that HPV testing in primary screening is unlikely to be cost effective since the population prevalence of HPV infection is over 15%. As a result, to ensure that women with significant CIN are not missed, many HPV positive women with no cervical neoplastic abnormality detectable by cervical cytology would be followed up unnecessarily. Even if HPV testing was restricted to women aged 35 or over to improve the specificity obtained and the screening interval was increased for women considered to be at low-risk (negative for both cytological neoplastic abnormality and for HPV), HPV testing in primary screening may still not be financially viable. However, this issue would be addressed by larger randomised trials such as the UK-based five centre screening trial of HPV testing with routine Pap cytology in 12,000 women aged 30 or older (the HART Study) (Cuzick, 2001). Evidence from the work described here suggests that combined LBC and HPV testing using HCAII on a single specimen would be cost-effective for the management of women with borderline smears because this approach would result in a reduction in the number of repeat cervical scrapes and colposcopy referrals.

When assessing the potential circumstances in which HPV testing may be of value, it is important to consider screening separately from diagnosis, since the types of HPV tests employed are likely to differ. For example, for routine screening of large numbers of clinical specimens, automated DNA extraction followed by real-time PCR using GP5+/6+ primers would be suitable for detection of a broad range of HPV types in LBC samples. However, for diagnosis and effective clinical management of immunosuppressed women or those with persistent CIN, assessment of HPV genotype or viral load would be a more useful tool. The work presented here suggests that there is a lack of reliability using detailed melting temperature analysis of real-time PCR products for HPV genotyping and therefore other methods are

more appropriate for clinically reliable HPV genotyping, such as hybridisation-based techniques. It is likely that hybridisation probe technology may be appropriate to distinguish accurately between the 30 or so known anogenital HPVs. In addition real-time PCR could be used to develop a quantitative system for assessment of HPV viral load by controlling for variation in the cellular content of the cervical sample by quantification of a host cell gene, such as β -globin, or using probe based techniques. Since PreservCyt®-fixed cells are also suitable for RNA extraction (Tarkowski *et al.*, 2001), RNA analysis of specific genes such as E6 and E7 (Molden *et al.*, 2001; Bahrani-Mostafavi *et al.*, 2001) may allow differentiation between latent and productive infection, and identify those women who would be more likely to develop progressive cervical disease due to active gene expression. To elucidate the clinical significance of multiple HPV infection, delineation of those HPV genotypes most frequently associated with cervical neoplasia progression is required. The RLBA may also prove useful for this purpose since it is less labour intensive than direct DNA sequencing which was of limited use for the identification of multiple HPV infections. Additionally some of the novel HPV genotypes require further characterisation so that molecular assays can be adapted to include them if they are considered to have oncogenic potential.

Additional approaches that may be implemented to improve prospective cervical screening programmes include the following. Use of automated cytology that would require high quality cytological preparations without overlapping cells or mucus such as those provided by LBC. The development of novel assays that detect other markers of cervical carcinogenesis such as markers of cellular proliferation (e.g. Ki67 or Mcm-2). The use of self-sampling devices to improve population coverage and increased screening intervals for HPV negative women with no cytological abnormality who are considered to be of limited risk.

This study contributes significant evidence on the usefulness of LBC within the Scottish cervical screening programme. The use of a variety of tests for HPV detection provides some direction for future studies where HCAII could be considered for screening purposes and HPV genotyping may be useful in specific clinical situations. Implementation of LBC and HPV testing may help provide

significant cost savings to the screening programme while improving female health by earlier detection of cervical neoplasia. Thus, such improvements may help reduce the morbidity and mortality rates associated with cervical cancer to an all-time low.

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zur Hausen H. (1996) Papillomavirus infections—a major cause of human cancers. *Biochimica et Biophysica Acta* **1288**: F55–F78. [

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APPENDIX 1

Solutions and Laboratory Media

Earles Balance Salt Solution

90ml sterile water
10ml Earles balanced salt solution (10×)
10ml new born calf serum
1ml amino acids
1ml vitamins
1ml glutamine
3ml 4.4% sodium bicarbonate
0.3ml penicillin/streptomycin (added prior to use)

GTC-containing Lysis Buffer

Dissolve 120g guanidinium thiocyanate in 100ml of 1M Tris-HCl, pH6.4. Add 22ml of 0.2M EDTA adjusted with NaOH to pH8 and 2.6g of Triton-X-100. Homogenise.

GTC-containing Wash Buffer

Dissolve 120g guanidinium thiocyanate in 100ml of 1M Tris-HCl, pH6.4.

50× TAE

242g Tris Base
57.1ml glacial acetic acid
100ml 0.5M EDTA
Make up to one litre.

1× TE Buffer, pH 7.4

10mM Tris-HCl (pH 7.4)
1mM EDTA (pH 8.0)

SOC Medium (100ml)

2g Bacto®-tryptone
0.5g Bacto®-yeast extract
1ml 1mM NaCl
0.25ml 1M KCl
1ml 2M Mg²⁺ stock (1M MgCl₂·6H₂O, 1mM MgSO₄·7H₂O), filter sterilised
1ml 2M glucose, filter sterilised

Add Bacto®-tryptone, Bacto®-yeast extract, NaCl and KCl to 97ml deionised water. Stir to dissolve. Autoclave and cool to room temperature. Add 2M Mg²⁺ stock and 2M glucose stock, each to a final concentration of 20mM. Filter the complete medium through a 0.2µm filter unit. The pH should be 7.0.

LB Medium (1L)

10g Bacto®-tryptone
5g Bacto®-yeast extract
5g NaCl

Add deionised water to approximately 1 litre. Adjust pH to 7.5 with 10N NaOH and autoclave.

APPENDIX 2

PATIENT CONSENT

CONSENT FORM

Title of Research: The presence and significance of human papillomavirus (HPV) infection in women infected with HIV

Name and address of Investigators: Dr Gerald Beattie M.R.C.O.G., Consultant Obstetrician and Gynaecologist, St Johns Hospital at Howden, Livingston EH54 6PP. Dr Heather A Cubie, Consultant Clinical Scientist, Regional Virology Laboratory, City Hospital, Edinburgh EH10 5SB

Further information available from: Dr David Farquharson, Consultant Obstetrician & Gynaecologist, Royal Infirmary of Edinburgh Tel: 0131 536 4247. Dr Farquharson is totally independent of this study.

- I have read and understood the accompanying information sheet and had the opportunity to ask questions about it.
- I understand that I have the right to withdraw from this study at any stage and that to do so will not affect my treatment.
- I agree to participate in the above study. I understand that my sample will be retained for research purposes.
- I understand that this is non-therapeutic research from which I cannot expect to derive any benefit.

Name of patient

Signature of patient Date

Signature of Doctor Date

Three copies: first copy retained by investigator, second copy by patient, third copy by General Practitioner.

CONSENT FORM

Title of research: *Liquid based cytological specimens for the detection of micro-organisms.*

Name and address of Investigator: *Dr E. McGoogan, Department of Pathology, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG; Tel 0131 650 2902.*

Further information available from: *Dr A. Glasier, Family Planning and Well Women Clinic, 18 Dean Terrace, Edinburgh EH4 1NL Tel: 0131 332 7941*

- I have read the information sheet and consent form and had the opportunity to ask questions about them.
- I agree to participate in this study. I understand that the aim is to try to improve the accuracy of the cervical smear test.
- I understand that participation in this study will not affect my treatment.
- I understand that the residual material from the sample will be used for research purposes and some may be retained for future use.
- I understand that I am under no obligation to take part in this study.
- I understand that this is non-therapeutic research from which I cannot expect to derive any benefit.

Name of patient

Signature of patient

Date

Signature of Smear Taker

Signature of investigator

Three copies: white copy retained by patient; pink copy sent to investigator; yellow copy to General Practitioner.

APPENDIX 3

ORAL AND POSTER PRESENTATIONS

Oral Presentations

Seagar AL.

DNA extraction from liquid-based cytology specimens.
QIAGEN Seminar, 22 November 1999, Glasgow.

Whitehead J, **Seagar AL**, Cubie HA, Fuerst RAUA, Arends MJ.

Rapid real time PCR to distinguish between high risk human papillomavirus types 16 and 18.

Scottish Diagnostic Virology Group, 21 May 1999, Glasgow.

Seagar AL, McGoogan E.

Improved detection of cervical neoplasia using the ThinPrep Pap Test and human papillomavirus DNA testing.

Abstract 117, Pathological Society of Great Britain and Ireland, 180th meeting, 18-21 January 2000, London.

Seagar AL, McGoogan E, Cubie HA, Lockhart G, Pitt P.

Use of liquid-based cytology and HPV testing in the follow-up of women with equivocal smears.

4th International Multidisciplinary Congress, EUROGIN 2000, 5-9 April 2000, Paris.

Cubie HA, **Seagar AL**, McGoogan E, Arends MJ, Whitehead J, Brass A, Moore C, Whitley M (presented by Dr HA Cubie).

Real-time PCR for the detection of HPV in liquid based cytology samples.

Abstract 192, 18th International Papillomavirus Conference, 23-28 July 2000, Barcelona.

Poster Presentations

McGoogan E, **Seagar AL**, Cubie HA, Pitt P, Whitehead J.

Detection of human papillomavirus in archival smears using Hybrid Capture Assay II.

Abstract Dia 25, 17th International Papillomavirus Conference, 9-15 January 1999, South Carolina, USA.

Beattie GJ, **Seagar AL**, Cubie HA, Monaghan S.

A longitudinal study of HPV detection and cervical pathology in HIV-infected women.

Abstract PVPF 30, 17th International Papillomavirus Conference, 9-15 January 1999, South Carolina, USA.

McGoogan E, Cubie HA, **Seagar AL**, Lockhart G, Pitt P.
Improved management of women with low-grade cervical smears using the ThinPrep Pap Test and human papillomavirus (HPV) DNA testing.
Abstract 256, 18th International Papillomavirus Conference, 23-28 July 2000, Barcelona.

Seagar AL, Arends MJ, Cubie HA, Stirling D, White AW.
DNA sequence analysis to identify human papillomavirus types in women infected with HIV.
Abstract 414, 18th International Papillomavirus Conference, 23-28 July 2000, Barcelona.

Cubie HA, **Seagar AL**, Beattie GJ, Arends MJ.
Detection and persistence of HPV in HIV infected women.
Abstract 185, European Virology 2000, 17-21 September 2000, Glasgow.

Cubie H, **Seagar AL**, McGoogan E, Arends MJ, Whitehead J, Brass A, Whitley MW.
Liquid based cytology samples and real time PCR for the detection of HPV within a cervical screening programme.
Abstract 186, European Virology 2000, 17-21 September 2000, Glasgow.

Cuschieri KS, **Seagar AL**, Moore C, Cubie HA.
Development of an automated extraction procedure for detection of human papillomavirus (HPV) DNA in liquid based cytology samples.
Abstract P-17, 19th International Papillomavirus Conference, 1-7 September 2001, Florianópolis, Brazil.

Cubie HA, McGoogan E, Cuschieri KS, **Seagar AL**, Moore C, Gilkisson G, Whitley MW.
Comparison of real-time PCR and reverse hybridisation Line Probe Assay for detection of HPV in LBC samples from routine cervical screening clinics.
Abstract P-6, 19th International Papillomavirus Conference, 1-7 September 2001, Florianópolis, Brazil.

Cubie HA, Cuschieri KS, McGoogan E, **Seagar AL**, Moore C, Gilkisson G, Arends MJ, Whitley MW.
Detection of HPV in liquid-based cytology (LBC) samples using real-time PCR and hybridisation. Abstract 5, Society for General Microbiology Workshop on Papillomaviruses, 150th Ordinary Meeting, 8-12 April 2002, Warwick.

APPENDIX 4

PUBLISHED PAPERS

Cubie HA, **Seagar AL**, Beattie GJ, Monaghan S, Williams ARW. (2000) A longitudinal study of HPV detection and cervical pathology in HIV infected women. *Sexually Transmitted Infections* **76**: 257-261.

Cubie HA, **Seagar AL**, McGoogan E, Whitehead J, Brass A, Arends MJ, Whitley MW. (2001) Rapid real time PCR to distinguish between high risk human papillomavirus types 16 and 18. *Molecular Pathology* **54**: 24-29.

Rapid real time PCR to distinguish between high risk human papillomavirus types 16 and 18

H A Cubie, A L Seagar, E McGoogan, J Whitehead, A Brass, M J Arends, M W Whitley

Abstract

Aims—To assess the validity and practicality of real time polymerase chain reaction (PCR) for human papillomavirus (HPV) testing in combination with liquid based cytology samples for cervical screening.

Methods—Real time PCR using consensus (GP5+/6+) and type specific primers was developed to detect genital HPV types. This provides rapid, efficient amplification followed by denaturation of the product and computer analysis of the kinetics data that are generated. Liquid based cytology samples were obtained from patients attending routine cervical screening clinics. DNA was extracted from the residual cellular suspension after cytology using spin columns.

Results—Real time PCR successfully distinguished between HPV-16 and HPV-18 on the basis of amplification with consensus primers followed by DNA melting temperature (T_m) analysis. Sensitivities of one to 10 copies of HPV-16 (mean $T_m = 79.4^\circ\text{C}$; 2 SD, 0.8) and four to 40 copies of HPV-18 (mean $T_m = 80.4^\circ\text{C}$; 2 SD, 0.4) were obtained. In a mixed population of SiHa and HeLa cells containing known copy numbers of HPV-16 and HPV-18 genomes, HPV-16 and HPV-18 products were clearly separated by T_m analysis in mixtures varying from equivalence to 1/1000. Together with detailed melt analysis, type specific primers from the same region of the L1 gene confirmed the differential ability of this system. The method was applied to 100 liquid based cytology samples where HPV status using conventional GP5+/6+ PCR was already known. There was 95% agreement between the methods, with 55 positives detected by conventional PCR and 59 with real time PCR. The method was then tested on 200 routine liquid based cytology samples. Approximately 10% were positive by real time PCR, most of which were classified as HPV-16 by detailed melt analysis. Thirteen (6.8%) HPV positives were identified in 189 samples showing no evidence of cervical cytological abnormality.

Conclusions—Real time PCR is a rapid, efficient method for the detection of HPV with the separation of HPV-16 and HPV-18 on the basis of differential T_m . Preliminary results suggest it could prove useful if HPV testing is added to cervical screening programmes.

(*J Clin Pathol: Mol Pathol* 2001;54:24-29)

Keywords: real time polymerase chain reaction; cervical screening; human papillomavirus types 16 and 18

More than 40 types of human papillomavirus (HPV) infect the genital epithelium and several high risk types including HPV types 16, 18, 31, 33, and 45 are found in almost all cases of high grade cervical intraepithelial neoplasia and cervical cancer.^{1,2} In Europe, the most prevalent type is HPV-16,¹ but there are several reports that HPV-18 infection can lead to the development of more clinically aggressive disease.³⁻⁵

Laboratory diagnosis of HPV infection is dependent upon molecular techniques such as DNA hybridisation or nucleic acid amplification. Several polymerase chain reaction (PCR) methods have been developed to detect a broad spectrum of mucosotropic HPV types using either degenerate or consensus primers.⁶⁻⁹ A second generation commercial hybridisation assay, Hybrid Capture™ (HCA II), is also available for the detection of HPV DNA in cervical swab samples,¹⁰ and has been used widely in epidemiological studies.¹¹⁻¹³ However, both consensus PCR and HCA II have important limitations. They are costly and labour intensive and, without additional procedures, neither technique can differentiate between individual types or detect infection with more than one type. Furthermore, HPV infections are often transient, frequently cleared by immunocompetent people, and require interaction with cofactors for the progression of disease. Thus, the development of highly sensitive detection tests for high risk HPV raises problems of clinical interpretation.

The potential use of HPV testing in cervical screening programmes is dependent on a rapid sensitive test that can distinguish high risk HPV types present in clinical samples. In most conventional PCR assays, amplification is performed by automated temperature cycling, but product analysis requires a subsequent manual operation.

Rapid real time PCR can distinguish closely related sequences on the basis of amplification followed by DNA melting temperature analysis. The commercial LightCycler (Idaho Technology Inc, supplied by BioGene Ltd, Kimbolton, Cambridgeshire, UK) combines simultaneous PCR amplification with sophisticated computer analysis of the kinetics data generated. The use of air as a circulating medium during PCR cycling allows rapid temperature control and thus a significant reduction in testing time (typically 40 cycles in 25 minutes). The use of fine capillaries of borosilicate glass provides efficient heat transfer and by acting as wave guides facilitates sen-

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Accepted for publication
17 October 2000
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sitive fluorimetry and enhances the efficiency of the amplification. The amplification mix contains a fluorescent dye, SYBR Green I™, which binds to the minor groove of double stranded DNA and emits light on excitation.¹⁴ Thus, as the PCR product accumulates, fluorescence increases. On denaturation of the product, SYBR Green I is released and fluorescence rapidly decreases. Because the melting curve of DNA is dependent on sequence, length, and GC content, PCR products can be distinguished by their melting curves. The determination of melting curves can be carried out on each sample after amplification without opening the reaction vessels.

We report the differentiation of HPV-16 and HPV-18 in mixed cell populations using GP5+/6+ consensus primers,⁹ with confirmation using type specific modifications of these primers, and the application of this method to clinical specimens.

Materials and methods

POSITIVE CONTROL MATERIAL

HPV containing cell lines

The cell lines, SiHa and HeLa, which contain one to two copies of HPV-16 DNA and 30–40 copies of HPV-18 DNA/cell, respectively,¹⁵ were grown as monolayers and passaged at regular intervals using standard cell culture techniques. Cells were removed from the plastic with gentle trypsinisation, counted, and the DNA extracted from a known number of cells in 200 µl of phosphate buffered saline (PBS) using spin columns (Qiagen DNA mini kit; Qiagen Ltd, Lewes, West Sussex, UK) in accordance with the manufacturer's tissue protocol.

Cloned material

Cloned DNAs of HPV types 16, 18, and 45 originally isolated from clinical material were provided by Professor E-M de Villiers (Referenzzentrum für humanpathogene Papillomviren, Heidelberg, Germany) and HPV-33 was obtained from Dr G Orth (Institut Pasteur, Paris, France). HPV types 16, 18, and 33 were provided in pBR322 and HPV-45 in pGEM4. HPV-31 was cloned in pT713 and was obtained from Dr A Lorincz (Digene Diagnostics, Silver Spring, Maryland, USA).

Transformation was performed using Library Efficiency DH5α™ competent cells (Life Technologies, Paisley, Scotland, UK). Plasmid containing colonies were selected using L-Amp plates and were then cultured in L-broth containing ampicillin. Plasmid DNA was isolated from this bacterial cell culture using the Hybaid recovery quick mini spin kit

(Hybaid Ltd, Middlesex, UK) according to the manufacturer's instructions. The extracted DNA was digested with selected restriction enzymes (BamHI, EcoRI, HindIII, HindII, and BglII) and electrophoresed in 1% agarose to check the identity of the DNA. Finally, GeneQuant II spectrophotometric analysis (Amersham Pharmacia Biotech, St Albans, Hertfordshire, UK) was performed to measure the concentration of DNA.

CLINICAL SAMPLES

A total of 300 liquid based cytology samples were collected from women attending general practitioner clinics for routine cervical screening or follow up. Cells were collected from the cervix using a Cervex Brush® rinsed in 20 ml of PreservCyt® (PC) solution (Cytoc Corporation; Boxborough, Massachusetts, USA). The sampler was then discarded. One hundred specimens were "split samples", where a conventional smear had been made before the residual cervical material was rinsed into the liquid based cytology medium. The other 200 samples were collected routinely and only a ThinPrep® (TP) monolayer smear was made for diagnostic purposes.

DNA EXTRACTION

Cells from the residual volume were pelleted at 2900 ×g for 15 minutes before resuspending in 200 µl Tris EDTA (TE) buffer (pH 7.2). DNA extraction was then performed directly using the Qiagen DNA mini kit, according to the manufacturer's tissue protocol, resulting in 400 µl of extracted sample.

PRIMERS AND PCR PROTOCOL

Three primer pairs were used in our study: the GP5+/6+ consensus primer pair, together with HPV-16 and HPV-18 specific primer pairs, which were modified from GP5+/6+ (table 1). These were designed after a thorough search of the HPV sequence database (Los Alamos National Laboratory) in the GP5+/6+ primer target region of the L1 gene. The DNA sequence of all known HPV types was critically reviewed for optimal product length, annealing temperature, base variations between types, and the possibility of non-specific amplification.

Reaction mixes contained 0.5 µl of 5 µM forward and reverse primers, 1.25 µl of template nucleic acid, 0.25 µl of 1/1000 SYBR Green I, and 2.5 µl of master mix containing 4 mM MgCl₂ (Biogene Ltd) and TaqStart antibody (Sigma-Aldrich Co Ltd, Poole, Dorset, UK). The cycling profiles were optimised for each set of primers and are detailed in table

Table 1 Sequences of primers used and optimal cycling conditions found

| Name | Sequence | Number of cycles | Cycling conditions |
|--------|-------------------------------|------------------|--|
| GP5+ | TTTGTACTGTGGTAGATACTAC | 5 | 95°C for < 1 second 50°C for 3 seconds, 72°C for 3 seconds |
| GP6+ | CAACTTCATCCACGTTTACC | 35 | 85°C for < 1 second 55°C for 3 seconds, 72°C for 3 seconds |
| TS165+ | TTGTACTGTGTGTTGATACTACA | 5 | 95°C for < 1 second 59°C for 3 seconds, 72°C for 3 seconds |
| TS166+ | GAAAAATAAACTGTAAATCATATTCCTCC | 35 | 82°C for < 1 second 59°C for 3 seconds, 72°C for 3 seconds |
| TS185+ | TTTGTACTGTGGTAGATACCACT | 5 | 95°C for < 1 second 62°C for 3 seconds, 72°C for 3 seconds |
| TS186+ | GAAAAATAAACTGCAATTCATATTC | 35 | 85°C for < 1 second 62°C for 3 seconds, 72°C for 3 seconds |

Table 2 Detailed melt analysis of DNA extracted from SiHa and HeLa cells

| | Number of replicates | T_m with GP5+/6+ (mean T_m (2 SD)) | Number of replicates | T_m with TS primers (mean T_m (2 SD)) |
|------|----------------------|--|----------------------|---|
| SiHa | 20 | 79.1 (0.8°C) | 30 | 78.2 (0.4°C) |
| HeLa | 20 | 80.9 (1.0°C) | 30 | 80.4 (0.3°C) |

1. Detailed melt analysis was used for the accurate determination of the melting point of the amplified product. This consisted of a single cycle of 65°C for three seconds to 90°C for one second at a transition rate of 0.2°C/second, followed by measurement of the fluorescent signal at greater frequency.¹⁶ The entire assay including DNA extraction, PCR amplification, and melting temperature analysis can be performed in approximately three hours.

HYBRID CAPTURE ASSAY

A 4 ml volume of PC fluid was processed for the Digene HPV hybrid capture assay (HCA) according to the manufacturer's recommendations and using the second generation (HCA II) test. This is a sandwich capture hybridisation system using chemiluminescent signal amplification for the qualitative detection of 13 different high risk HPV types (HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68). The emission of light is measured as relative light units (RLUs) and is proportional to the amount of target DNA present. Samples with an RLU > 1 were considered positive for any of the high risk HPV types contained in the probe pool. HPV results were correlated with the cytology results obtained.

CYTOLOGICAL ASSESSMENT

TP slides were made using the semi-automated ThinPrep 2000 slide processor (Cytoc Corporation, Boxborough, Massachusetts, USA). The methodology has been well documented elsewhere.^{17,18} Both conventional and TP smears were reported independently. The Richart system for cervical diagnostic reporting was used (unsatisfactory (U/S), negative (WNL), borderline changes (B/L), mild dyskaryosis, moderate dyskaryosis, and severe dyskaryosis).

Results

CONTROL MATERIAL

Cultured cells containing HPV-16 or HPV-18

With GP5+/6+ primers, replicate 10-fold dilutions of SiHa cells containing 1000–2000 genome copies down to 0.1 copy of the HPV-16 genome were tested and the product gave a single peak and a sensitivity of 1–2 copies/μl. Similarly, 10-fold dilutions of HeLa cells containing 5000 down to 0.5 copies of the HPV-18 genome showed a sensitivity of 3–50 copies/μl. Detailed melt analysis of 20 samples each of SiHa and HeLa cells gave the mean T_m of the HPV-16 product as 79.1°C (2 SD, 0.8) and the mean T_m of the HPV-18 product as 80.9°C (2 SD, 1.0) (table 2). In a mixed population of SiHa and HeLa cells, a composite melting curve was observed on standard analysis, with a T_m of 79.4°C (fig 1A). On further analysis using a detailed melt cycle, this resolved into two clearly differentiated peaks with temperatures of 78.4°C and 80.1°C (fig 1B). Using this system, HPV-16 could be detected in a 1/1000 mixture of SiHa to HeLa cells.

Type specific primers, TS165+/166+ and TS185+/186+ (table 1), from a similar region of the L1 gene gave individual peaks at 79.2°C and 81.3°C for HPV-16 in SiHa and HPV-18 in HeLa cells, respectively, and detailed melt analysis of 30 samples confirmed these mean melting points as 78.2°C (2 SD, 0.4) and 80.4°C (2 SD, 0.3), respectively. The specificity of these primers led to a smaller standard deviation in T_m (table 2). Different cycling conditions were required for each set of specific primers, reflecting the optimal conditions for stringency of each pair. The differential ability of the specific primer pairs was assessed by amplifying mixtures of SiHa and HeLa cells in the presence of one or other primer pair. In mixtures with equivalent copy numbers, both types were readily detected. Again, HPV-16 from a single SiHa cell could be detected in a background of 10³ HeLa cells (table 3).

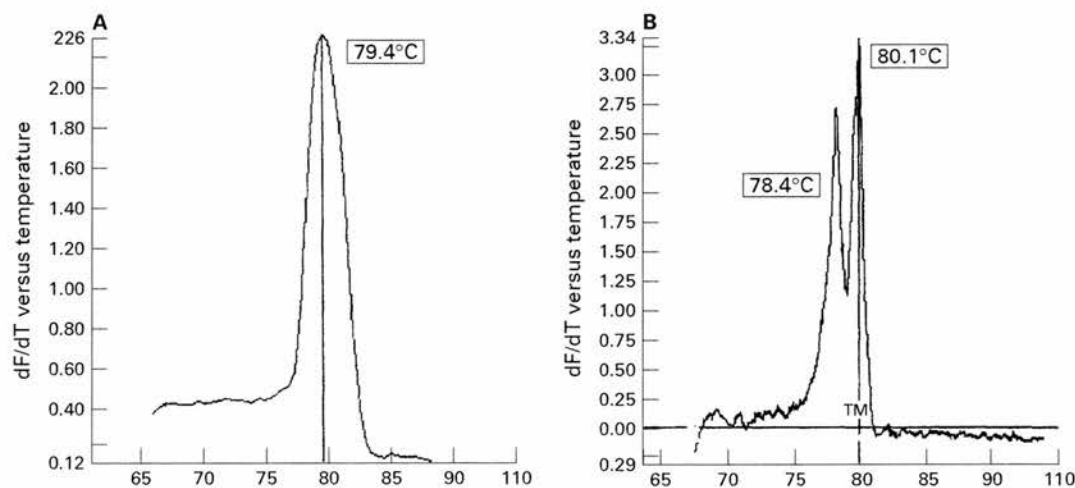


Figure 1 Melting curve analysis of PCR products after amplification of a 150 bp fragment of the human papillomavirus (HPV) L1 gene. Y axis: negative differential of fluorescence over temperature ($-dF/dT$). (A) Standard melt analysis of SiHa/HeLa mixed DNA product, using GP5+/6+ primers showing single peak ($T_m = 79.4^\circ\text{C}$). (B) Detailed melt analysis of SiHa and HeLa mixed DNA product, showing two peaks with $T_m = 78.4^\circ\text{C}$ (HPV-16) and 80.1°C (HPV-18).

Table 3 Detection of human papillomavirus 16 (HPV-16) in a background of HPV-18 with GP5+/6+ and with TS 165+/166+ primers

| SiHa/HeLa | GP5+/6+ | | | TS 165+/166+ | | |
|-----------|---------------------|---------------------|-----------------------|---------------------|---------------------|-------------|
| | Standard melt T_m | Detailed melt T_m | Comments | Standard melt T_m | Detailed melt T_m | Comments |
| 100/1000 | 80.9°C | 79.9/81.4°C | Double peak | 79.1°C | 78.2°C | Single peak |
| 10/1000 | 81.0°C | 79.9/81.5°C | Double peak | 79.1°C | 78.2°C | Single peak |
| 1/1000 | 81.5°C | 80.1/81.8°C | Double peak | 79.1°C | 78.1°C | Single peak |
| 0.1/1000 | 81.4°C | 80.3°C | Single peak of HPV-18 | No amplification | No amplification | No peak |

Table 4 Melting temperature (T_m) of high risk human papillomavirus (HPV) cloned material using real time PCR with GP5+/6+ primers and detailed melt analysis

| HPV type | T_m |
|----------|--------|
| HPV-33 | 76.7°C |
| HPV-31 | 78.0°C |
| HPV-45 | 78.2°C |
| HPV-16 | 79.4°C |
| HPV-18 | 80.4°C |

Table 5 Comparison of conventional and real time PCR on 100 selected clinical samples

| Conventional PCR HPV results | Light Cycler PCR HPV results | | | Total |
|------------------------------|------------------------------|-----------|----------|-------|
| | Positive | Equivocal | Negative | |
| Positive | 55 | 0 | 0 | 55 |
| Negative | 4 | 1 | 40 | 45 |
| Total | 59 | 1 | 40 | 100 |

HPV, human papillomavirus.

Cloned material

Detailed melt analysis allowed the differentiation of five different types of high risk HPV from cloned material and showed a range from 76.7°C for HPV-33 to 80.4°C for HPV-18 (table 4). We are currently investigating the differentiation by T_m of additional HPV types.

PATIENT SAMPLES

One hundred preselected samples previously analysed using HCA and conventional PCR were assessed using real time PCR. Table 5 shows the comparison between conventional and real time PCR. Five samples were positive by real time PCR but negative by conventional PCR. Of these five discrepant samples, one contained HPV-16 as shown by use of type specific primers from both the L1 and E6 regions,¹⁵ one contained an HPV-16 related type as shown by detection with HPV-16 E6 primers but not HPV-16 L1 primers, and three contained HPV "X", being positive by HCA but negative with HPV-16 L1 and E6 primers and also HPV-18 type specific primers.

The first 200 routine cervical screening samples from our study looking at the combined effectiveness of liquid based cytology sampling and real time PCR were processed within 21 days of receipt and analysed without

previous knowledge of the cytology results. Nineteen (9.5%) of the 200 samples contained HPV (table 6), of which 13 (6.8%) were found in women with no evidence of cytological abnormalities. Most of the positive samples (12 of 19) appeared to contain HPV-16 by detailed melt analysis (fig 2A) and an occasional double infection was noted (fig 2B).

Discussion

We have shown that HPV-16 and HPV-18 can be detected by rapid real time PCR using consensus primers and can be differentiated by melting curve analysis. In mixed samples, two separate peaks of distinct T_m are seen, even when there is a considerable difference in the copy number of each type. This approach has been confirmed with type specific primers.

Because HPV-16 and HPV-18 product lengths are very similar, it is impossible to differentiate between individual HPV types by agarose gel electrophoresis after GP5+/6+ PCR amplification. In contrast, melting curve analysis using LightCycler technology can distinguish between products of the same length but different GC:AT ratios.¹⁸ Woo and colleagues¹⁹ reported the use of genus specific amplification primers and specific fluorogenic hybridisation probes to differentiate pathogenic and non-pathogenic strains of leptospira. In their study, the lower limit of detection was 200 genome copies. The same group also used melting curve analysis to differentiate different strains of leptospira²⁰ and to distinguish reference strains and field isolates of leptospira without the use of hybridisation probes.²¹ More recently, reports of the use of the LightCycler in detecting viruses have begun to appear. These include the quantitative detection of human cytomegalovirus (CMV) in plasma²² and the sensitive diagnosis of herpes simplex virus in clinical samples using T_m analysis to distinguish between herpes simplex virus 1 (HSV-1) and HSV-2.²³ We have used a similar approach to separate HPV types, specifically for the differentiation of HPV-16 and HPV-18 products that differ in T_m by less than 2°C.

Real time PCR technology has great potential for clinical and non-clinical development. Nevertheless, it is still a new technique and some technical problems have been reported, including the presence of primer dimer formation.¹⁹ In our hands this could be minimised by careful attention to optimisation conditions, with very small changes in concentration, temperature, and the use of TaqStart antibody having a considerable effect on the shape of the analytical trace. In addition, the presence of a high molecular weight non-specific PCR product has been noted in some applications.¹⁹ Wit-

Table 6 Human papillomavirus (HPV) findings related to cytology in 200 unselected, routine liquid based cytology samples

| Cytology | Total number of samples | HPV positive | HPV negative |
|----------------------|-------------------------|--------------|--------------|
| Unsatisfactory | 2 | 0 | 2 |
| Negative | 189 | 13 (6.8%) | 176 |
| Borderline | 1 | 0 | 1 |
| Mild dyskaryosis | 7 | 5 | 2 |
| Moderate dyskaryosis | 0 | 0 | 0 |
| Severe dyskaryosis | 1 | 1 | 0 |
| Total | 200 | 19 (9.5%) | 181 |

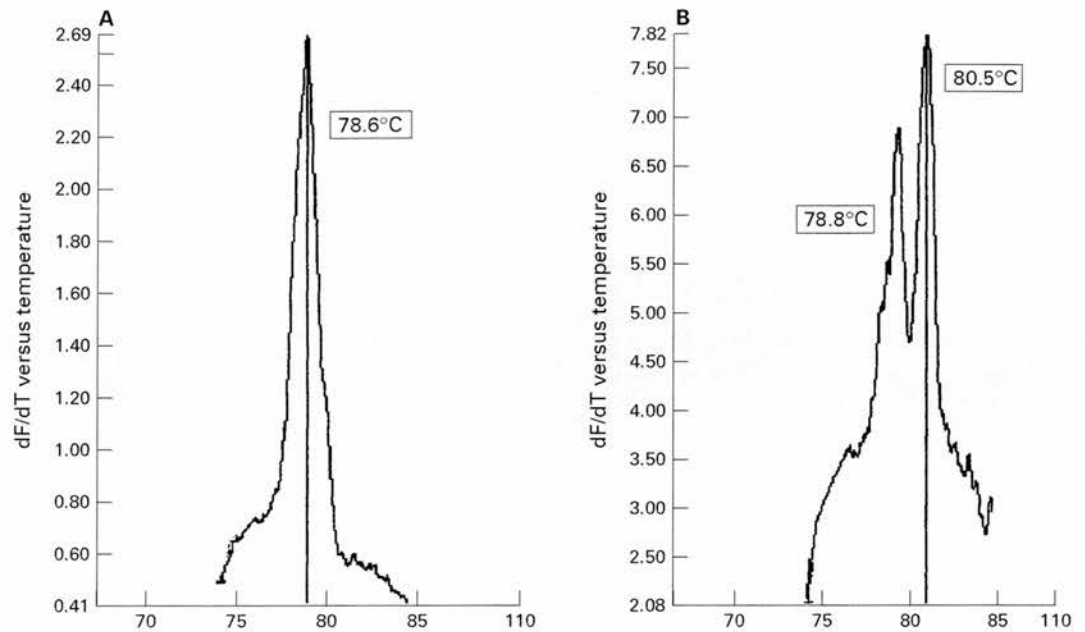


Figure 2 Melting curve analysis of PCR products from clinical liquid based cytology samples. (A) Detection of a single peak of human papillomavirus 16 (HPV-16) using detailed melt analysis. (B) Detection of a mixed infection with HPV types 16 and 18 using detailed melt analysis.

twer and colleagues¹⁴ suggested that "shoulders" of non-specific PCR product might be caused by substantial product to product annealing in later amplification cycles. A small shoulder was sometimes seen in our study on the high temperature side of the melting curve. The problem was minimised by reducing the amplification mix from 10 μ l to 5 μ l and indeed the manufacturers recommend a volume of 5–7 μ l as optimal in each capillary. Larger volumes can result in uneven temperature distribution and therefore inefficient amplification towards the top portion of the capillary. With optimal conditions and volume, we only observed this phenomenon occasionally with type specific primers and it did not interfere with the determination of the T_m value. The effect was never seen with the consensus primers. Quality control of the reagents used is undoubtedly important to limit variations in T_m , and positive control material was included and fully analysed in every run.

Additional practical problems have included the fragility of the capillaries. However, breakages were minimal with experienced operators and the second generation LightCycler produced by Roche Molecular Systems uses more robust capillaries. The extended use of the LightCycler for detailed melt analysis can result in overheating of the carousel and, in our hands, only 10 detailed melt analyses could be carried out before a 30 minute cooling period was required. Nevertheless, the rapid cycling time with standard melts allowed up to seven PCR runs to be carried out in a single working day.

We used melting point analysis after amplification in a single reaction for the detection of single and mixed samples of HPV-16 and HPV-18 in both cell lines in vitro and in cervi-

cal secretions from patients, including 100 samples validated by both conventional PCR and HCA II. Subsequently, the protocol was applied successfully to 200 liquid based cytology clinical samples, with HPV DNA being detected in 9.8% of samples (table 6), including 13 of 189 (6.8%) showing no cytological abnormality. Although this is consistent with other studies,^{11–24} analysis of the HPV results in relation to cervical dyskaryosis requires a much larger study group and this work is currently under way. The combined approach of rapid amplification and product identification in a single PCR reaction is an exciting one, with great potential for both clinical and non-clinical development, particularly in terms of introducing HPV testing into cervical screening programmes. Opportunities for high throughput are possible by combining one of several available robotic handling instruments for DNA extraction with the LightCycler system and we are testing the maximum daily capacity of such combinations.

We are grateful to Professor E-M de Villiers (Referenzzentrum für humanpathogene Papillomviren, Heidelberg, Germany) for providing HPV types 16, 18, and 45; Dr G Orth (Institut Pasteur, Paris, France) for HPV-33; and Dr A Lorincz (Digene Diagnostics, Silver Spring, Maryland, USA) for HPV-31. We acknowledge the help of Dr B Morris, department of pathology, University of Edinburgh with growth and isolation of the plasmid DNAs. We would like to thank the Chief Scientist Office of the Scottish Executive for funding this work (Grant No K/MRS/50/C2699) and the NHS R&D Support Fund for additional support.

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ERRATUM

Cubie HA, Seagar AL, McGoogan E, Whitehead J, Brass A, Arends MJ, Whitley MW. (2001) Rapid real-time PCR to distinguish between high risk human papillomavirus types 16 and 18. *Molecular Pathology* **54**: 24-29.

Error in Table 1

The GP6+ primer sequence should read GAAAAATAAACTGTAAATCATATTC

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Printed in Great Britain by Meridian Print Centre Ltd. Derby.

MP/Feb/01